Biological Response Modifiers:

Subcommittee Report

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Biological Response Modifiers:

Subcommittee Report

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Subcommittee on Biological Response Modifiers
Board of Scientific Counselors
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National Cancer Institute

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SECTION I

Overview and Recommendations

INTRODUCTION

In October 1978, the Division of Cancer Treatment, NCI, made the decision to organize a program for BRM. The Board of Scientific Counselors of the Division of Cancer Treatment established a Subcommittee on Biological Response Modifiers (table 1-1) to review existing clinical and laboratory experience in this area, to develop guidelines for developing the BRM versus potential therapeutic agents, and to provide recommendations for the establishment of the new Program. This Report describes the results of the deliberations of the Subcommittee. An initial Interim Report was submitted to Dr. Vincent DeVita, Jr., then Director of the Division of Cancer Treatment and to its Board on October 30, 1979.

Although the immediate goals of this program are therapeutic and many of the recommendations for initial studies reflect those goals, we must emphasize that the scientific bases of biological response modification must be explored extensively, so that 1) the arbitrary choices of preparation and dose are eliminated, 2) more specific and potent products are provided in the future, 3) the necessary information required for the optimal utilization of agents now available is also provided, and 4) novel approaches are developed.

Definition of Biological Response Modifiers

When we speak of BRM, we refer to the agents or approaches that will modify the relationship between tumor and host by modifying a host's biological response to tumor cells, with resultant therapeutic benefit. The modification may be by several mechanisms of which the following are only a few examples: 1) increase of host antitumor responses through augmentation of effector mechanisms or mediators of host defense or decrease in those components of the host reaction that may be deleterious to the host, 2) increase of host defenses by the administration of natural or synthetic effectors or mediators, 3) increase in the efficacy of host response through changes in the tumor cells that might stimulate a greater host response or an increase in tumor cell sensitivity to a response, 4) decrease in the development (transformation) of tumor cells and/or increase in their differentiation (maturation), 5) increase in the ability of the host to tolerate damage by cytotoxic modalities of cancer treatment.

A review of clinical and laboratory experience suggested that the following functionally grouped agents be considered for inclusion in the BRM Program: 1) agents which augment antitumor immunity, modulate components of the immune response, and induce or restore effectors of immunity; 2) IF and cytokine products; 3) thymic hormones and factors; 4) tumor-specific vaccines; 5) immune effectors in the form of cells or antibodies; 6) agents which interfere with carcinogenesis; and 7) agents which modify tumor clonogenicity and/or the maturation of tumor or stem cells. Although traditional chemotherapy and radiotherapy are not considered as representing BRM, their effects on host biological responses are recognized and should be considered in appropriate BRM studies. Moreover, BRM agents have been, and will often be, studied in conjunction with the other modalities of therapy. Surgery and surgical manipulations of the host are not considered in this Report, despite the fact that they may also affect the relationship between tumor and host; these treatments may best be placed under the domain of the surgical oncology research programs being formulated.

Historical Development of the Biological Response Modifier Area

During the past 20 years, scientists have accumulated sufficient knowledge of the biological interactions between tumor and host in laboratory and clinical studies to indicate that the BRM likely have a significant role in cancer treatment. The recognition and appreciation of the clinical potential of the complex BRM area have occurred slowly, as was true for chemotherapy in the 1930's and 1940's. Like chemotherapy in its formative years, therapeutic utilization of BRM still meets with considerable skepticism particularly as a result of certain initial frustrating and disappointing clinical experiences following premature and overoptimistic conclusions. Although researchers have received substantial support from different sources at NCI, no program was identified to foster the development of BRM in their broad perspectives, i.e., in the systematic way that chemotherapeutic agents have successfully been developed for the past 20 years.

The belief that BRM are likely to have a significant role in cancer therapy stems from progress achieved along several independent lines of research that were reviewed by the Subcommittee. Essential relevant knowledge acquired about BRM is mentioned below.

Following the initial observations in the early 1960's that syngeneic and autochthonous tumors are immunogeneic in mice, many investigators have attempted to show that human tumors are also specifically recognized as antigenic in the primary host. Although this issue has not been resolved conclusively, evidence exists that at least some human tumors carry tumor-associated cell surface antigens. Several types of immune mediators and effectors, including identifiable subsets of lymphocytes, cells of the monocyte–macrophage type, and NK cells, in specific and nonspecific responses, have been identified in animals and to some extent in man. Additional systems of host defense may be identified in the future and may also include nonimmunological systems, such as biological factors that affect the metastatic process. Although the reasons for the appearance and growth of a tumor are still under study, the quantitative or qualitative defect which is postulated to exist in the cancer patient might be correctable by an appropriate BRM with resultant therapeutic benefit.

The possibility of augmenting the host response through selective modification of various components of the response has been demonstrated in animals with various chemicals, natural products, or physiological factors. Some therapeutic benefits have been achieved by augmentation in animals. Although a beneficial effect in man is necessarily far more difficult for one to establish, some encouraging, though by no means definitive, results have been reported in patients with certain types of cancer. The results with such agents have suggested a prolongation of

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survival (with or without disease) and/or increase in the remission duration induced by conventional therapy, in some instances with concurrent evidence of augmentation or restoration of functional tests of host defense. Unfortunately, many incomplete or uncontrolled clinical studies with inadequate numbers of patients, particularly with BCG treatment, generated considerable skepticism about such immunotherapy. The clinical studies with augmenting agents were reviewed for identification of trials that can be evaluated and are well designed. On the basis of this review, we concluded that, under certain conditions, sufficient indications of promise justify pursuit of this area of clinical research, especially those with defined chemical components or structure.

Although the existence of IF as antiviral factors has been known for about 20 years and their antitumor action demonstrated in animal systems about 10 years ago, it is only recently that they have been purified to a substantial degree. Human IF have now become available in sufficient amounts for testing of their antitumor activity in humans. Recent observations indicate that IF from human leukocytes can induce tumor regression in some patients. Increased knowledge has been acquired recently about the structure of IF and their production and purification. In view of the information now available and the practical possibility that large quantities of IF can be obtained, their orderly clinical evaluation as antitumor agents is justified.

The serum of BCG-infected mice treated with endotoxin

contains a substance (TNF) that mimics the tumor-necrotizing action of endotoxin itself and appears to have the characteristics of a cytokine. Although TNF is not a residual endotoxin but a factor released from host cells, probably macrophages, if induced in the same way in rats and rabbits, it also causes necrosis of transplanted murine tumors. Unlike endotoxin, TNF is toxic in vitro for neoplastic murine and human cell lines but not for mouse embryo culture. It has striking effects on immunological reactions in vitro, some like and others unlike those of endotoxin. We are at various stages of our knowledge with other cytokines. The roles and functions of lymphocyte activation factor, tall growth factor, macrophage inhibitory factor, and others are being studied, and they too may have important therapeutic applications.

The role of thymic hormones and factors in the development of immune responses and in the restoration of responsiveness in immunodeficiencies has been well documented. The restoration of immune responsiveness has also been observed in some patients with cancer. This observation lends support to the hypothesis that such restoration may aid the cancer patient who may be immunosuppressed as a result of cytoreductive chemotherapy and/or the disease itself. Considerable progress has been made in the purification of some of the thymic hormones, in the identification of their chemical structures and specific role in the immune system, and, recently, in the synthesis of 3 of these factors. Thus immune restoration by thymic hormones and factors, as an adjunct to cancer chemotherapy, is an approach ready for systematic clinical evaluation.

Considerable information has been accumulated in the area of the so-called tumor-specific treatments which include antibodies, immune cells, and/or macrophages activated specifically against TAA. The possibility of our achieving specific immunotherapy has been enhanced recently by 1) the demonstration that in certain cases TAA are recognized by the human host and 2) the opportunities we have to utilize monoclonal antibodies obtained through the use of hybridomas. In addition, recent progress in the use of immune cells for tumor immunotherapy models, cloning of cytotoxic animal and human lymphocytes, and the use of marrow transplantation as an adjunct for chemoradiotherapy for neoplasia in man justifies increased investigations of these potentially therapeutic approaches. This area should be vigorously pursued with developmental studies, so that the unique tumor specificity of the immune responses may ultimately be exploited.

With the recent observations that retinoids retard the onset of experimental tumors in animals, clinical evaluation of chemoprevention has been initiated. One demonstration that the expression of carcinogenic transformation is modified by these compounds indicates that this type of BRM may provide therapeutic opportunities in patients with high risk for secondary disease or perhaps in patients with high risk for recurrent disease.

Significant progress has been made in experimental model systems in identification of physiological factors regulating the maturation of normal stem cells, particularly from the bone marrow, and the clonogenicity of tumor cells. Studies in these areas should be encouraged and should include consideration of the possibility that some of the cytotoxic anticancer agents known to have effects on DNA structure or function may also act, at least in part, through a favorable modification of "maturation" mechanisms in target tumors.

After about two decades of independent and seemingly unrelated lines of investigation, it is becoming clear that the relationship between tumor and host may be modified through a variety of approaches. Some of these need increased attention at the basic research and preclinical levels, whereas others are ready for systematic and careful clinical evaluation.

General Characteristics of Biological Response Modifiers

As defined above, BRM are expected to increase the efficacy of the host response against tumor. Because these responses are known to be most effective against a small tumor load and ineffective against a large tumor, in most cases it may not be possible, feasible, or reasonable for one to test the antitumor efficacy of these agents in man by direct measurement of tumor regression. In fact, one might expect this group of agents to be most effective against tumors clinically undetectable but destined to recur. Accordingly, BRM are often likely to be most effective if administered as an adjunct to cytoreductive therapy especially after clinical remission has been induced by such therapy. Such an approach is also justified by the fact that cytoreductive therapy may have untoward toxicity on the host targets of BRM action and that BRM may potentially reduce some of the damaging effects of cytoreductive treatment. However, the usefulness of BRM need not be restricted to the treatment of minimal residual disease.

In the past, BRM have been used primarily in conjunction with other modes of treatment and only moderate measurable antitumor effects have been reported, with prolongation of survival (with or without disease). It is anticipated that the parameters of functional augmentation of host responsiveness measured to monitor the effects of BRM therapy may provide a correlate to antitumor action.

Some of the BRM may be expected to induce a cascade of indirect effects with different degrees of amplification. Thus BRM are not likely to exert effects that may be measured by the customary criteria used to measure dose-related cytotoxicity of drugs or radiation. Indeed, in most cases, the monitoring of specific functional parameters of host response and measurements of disease-free interval and survival may provide the only realistic quantitation of BRM efficacy. The intralesional and intracavitary administration of nonspecific augmenting agents (such as BCG) and current studies with systemically administered IF may represent important exceptions. It may be more appropriate for us to expect to detect measurable direct antitumor effects of BRM in the future, especially as more BRM become available in purified form. Of course, given some of the known selective effects of some anticancer agents on host responses, some anticancer drugs, presumed to act only through their antiproliferative and cytotoxic action, may also act partly through BRM mechanisms.

Finally, because BRM are expected to act through physiological mechanisms, they may often exert only moderate toxicity, and such toxicity may not be directly related to the BRM effect.

OVERVIEW OF PROGRAM COMPONENTS

The components of the BRM program as formulated to date include the following: 1) IF and IF inducers; 2) thymic hormones and factors; 3) agents which augment, modulate, or restore immunological reactivity; 4) TAA; 5) antitumor immune cells; 6) antitumor antibodies; 7) tumor preventive agents; 8) cytokines; and 9) maturation and differentiation factors. An overview of each of these components is presented below. The components are comprehensively reviewed in Sections II–VIII and in the workshop summaries presented in Section IX.

Interferons and Interferon Inducers

Since 1957 when the IF were discovered, interest in them has waxed and waned. Recently, interest has resurged in the potential therapeutic uses of IF stimulated by encouraging laboratory and clinical results. In one of a series of recent editorials in *The New England Journal of Medicine* in 1979, Dr. Thomas Merigan, a pioneer in this area, states that "the positive results achieved in viral infections and in tumor experiments in animals and in the limited human trials so far completed, suggest it could be one of the first natural products for use in human medicine, with an effect potentially as great as that of the corticosteroids."

The IF are inducible glycoproteins produced by cells in response to viruses and other stimuli, such as bacteria, protozoa, some low-molecular weight substances like cycloheximide and kanamycin, and high-molecular weight materials, such as double-stranded RNA. When lymphocytes are stimulated by antigens or mitogens, Type 2 IF is also produced. The IF are relatively species specific, i.e., an IF produced in mice will have activity largely, if not solely, on mouse tissue and not on human tissue. The IF induce resistance to a broad spectrum of viruses. This has been documented in vitro and in animals with the use of many tumor and non-tumor-inducing viruses. The mechanism of the antiviral resistance induced involves the binding of the IF molecules to receptors on a cell surface and consequent synthesis of 3 proteins by the cell that act to prevent viral reproduction.

Also greatly affected by IF is the immune response. Extensive studies in vitro and in animals have revealed a variety of effects of B- and T-cell functions and on NK cells and macrophages with enhancement or suppression of a given response partly depending on the timing of IF treatment, the antigens involved, and the particular response studied. For example, IF treatment of responding lymphocytes in vitro or in vivo inhibits the proliferative responses to mitogens or alloantigens. On the other hand, the specific cytotoxic T-lymphocyte response and the activity of NK cells is enhanced by IF. As more sophisticated tools for dissection of responses to tumor and nontumor antigens become available, the more precise sites and mechanisms of action of IF on immune response components will be better analyzed and identified.

Finally, IF affect the growth of various virus-induced primary and transplantable tumors in animals. The antitumor effect, which has been variable and usually modest, has occurred more often when the tumor load was small, when IF were administered repeatedly, and when they were used as an adjunct to chemotherapy. The mechanism of the antitumor effects has not been determined. We know IF inhibits the division of normal and malignant cells in culture and prolongs the interval between rounds of cell division. In addition to having an antiproliferative effect, however, IF affects the cell surface structure by increasing expression of cell surface antigens, strengthening the cytoskeleton, and modifying the phenotypic expression of cell surface characteristics. Its antitumor effects may reflect one or all of the above-mentioned activities and/or the modulation of the host response to the tumor with, e.g., stimulation of macrophage, NK, or cytotoxic T-cell function.

Studies in man have also documented effects of IF on virus infections, immune responses, and cancer. Antiviral therapy with parenteral administration of HuLeIF has recently yielded encouraging results. In 3 of 3 patients with chronic active hepatitis and hepatitis B infection, IF administration was associated with a rapid and reproducible fall in all Dane-particle markers. Moreover, controlled studies have demonstrated that HuLeIF decreases the progression and dissemination of herpes zoster in cancer patients, promotes the healing of herpes simplex virus interstitial keratitis, and reduces the incidence of herpes labialis reactivation after trigeminal ganglion decompression. Herpes zoster is, of course, a major problem in immunologically suppressed patients. Finally, a study in immunosuppressed renal transplant recipients showed that prophylactic IF delayed the shedding of CMV and decreased the incidence of viremia after transplantation. This is important for recipients of kidney transplants and potentially of even greater importance for recipients of allogeneic marrow transplants who exhibit an inordinately high incidence of fatal CMV-induced pneumonitis.

Although many of the various effects on immunologic responses in man in vitro and in vivo that have been reported are preliminary, human IF have enhanced T-cell cytotoxic responses and NK cell activity against tumor and virus-infected targets. This area of research is in an early stage and should be promoted with increased support.

Recently, preliminary data have been presented at meetings or, less frequently, in publications, that suggest a therapeutic anticancer effect of systemically administered HuLeIF. Encouraging results have been reported in patients with osteogenic sarcoma, multiple myeloma, nodular poorly differentiated lymphocytic lymphoma, and breast cancer. In Sweden, patients with osteogenic sarcoma received HuLeIF as adjuvant therapy after surgical resection of their primary tumor. The incidence of metastatic recurrence was reported to be lower in the IF-treated group of 38 patients compared with 35 historical controls (treated in 1952–72) and 30 "contemporary" controls. Unfortunately, although this study did not include prospective randomized controls, the results are encouraging and are awaiting critical statistical analysis. No reports of IF effect that can be evaluated against measurable osteosarcoma are available.

The Swedish group also observed 5 partial and 3 complete responses among 16 patients with multiple myeloma who were given HuLeIF. Similar responses were

obtained in 6 of 10 patients with multiple myeloma treated with HuLeIF at M. D. Anderson Hospital and Tumor Institute, but far more modest results were reported by others.

Some non-Hodgkin's lymphomas also have been reported to respond to HuLeIF. At Stanford University Medical Center, 3 of 3 patients with indolent nodular poorly differentiated lymphocytic lymphoma exhibited a significant tumor regression in response to IF as did 6 of 11 lymphoma patients treated at M. D. Anderson Hospital and Tumor Institute. Interestingly, no significant effects were noted in patients with histiocytic lymphoma.

Significant antitumor activity was also observed in patients treated with HuLeIF for breast cancer. Of 17 patients initially treated at M. D. Anderson Hospital, 7 exhibited some degree of objective response. Other preliminary data suggest that various regimens of HuLeIF have some antitumor effect against a few patients with ALL as reflected by a significant reduction in the peripheral blood blast count and, to a lesser extent, in the marrow. Studies are in progress around the world on patients with Hodgkin's disease, myeloma, breast cancer, melanoma, lymphoma, AML, lung cancer, cancer of the stomach, hepatoma, and osteogeneic sarcoma. Overall, the most recent results presented at meetings have been far less positive than those discussed above.

Nevertheless, the data available for critical review demonstrate convincingly that HuLeIF has *some* objectively measurable antitumor effects in *some* patients with *some* types of cancer. However, insufficient data preclude any conclusions about the overall efficacy of such therapy and the potential role of IF as a therapeutic modality alone or as an adjunct to other therapies. It is tempting for us to hope that, if the anticancer therapeutic results observed were obtained with variable and potentially suboptimal regimens empirically derived without the benefit of classic phase I trials and with relatively crude material, then results in the future may reveal greater therapeutic efficacy when more purified IF is administered in a dose and schedule considered optimal on the basis of phase I trials. This view, of course, assumes that the positive effects of the present preparations do not reflect a *beneficial* one of contaminants.

The HuLeIF used in cancer therapy has been tolerable, with modest toxicity. The toxicity may or may not increase as the purity of the IF used is increased.

The mechanism for the anticancer effects observed in man is not known, but it may reflect the antiproliferative activity of IF, its effects on the cell surface of the tumors, and/or its effects on one or another component of an immune response to the tumor. The mechanisms must be determined because they will have major implications on how and when IF can be used in conjunction with an immunosuppressive chemotherapeutic antitumor agent. Accordingly, the effects of IF on various components of the immune response must be better defined in appropriate model systems.

Although the major clinical cancer therapy studies have been performed with IF obtained from leukocytes by the method of Cantell, human IF is also available from fibroblasts, but this latter product is costly, antigenically different from the leukocyte material, less stable, tends to stick to proteins at the site of injection (consequently, it does not enter the blood circulation in large amounts), and does not achieve the same active serum levels compared with that achieved with leukocyte IF unless it is given iv. A third source for human IF is represented by human lymphoblasts containing an integrated EBV genome, such as the Namalva strain of a Burkitt's lymphoma cell. The major reservations revolve around the unknown risk posed by the EBV genome or its product, if it should persist in the final product despite purification. A method has already been developed for removal of virtually all viral nucleic acids and contaminating protein from the final IF product; the material is undergoing clinical trials.

Because the critical impediment to the full use and study of IF has been the lack of the expensive material, two other approaches are being explored for the production of a far less expensive and more pure human product. The first consists of determination of the sequence of amino acid units of HuLeIF with a view of synthesis. The second, and recently reported as successful, is the application of the recombinant DNA techniques so that the IF gene can be introduced into bacteria and yeast which could then serve as IF factories.

An indirect approach to the problem has been the use of IF inducers in the form of synthetic double-stranded polyribonucleic acids. In animal models, inducers have had many effects on immunologic reactivity and on tumors. The advantages of these agents would be the following: 1) cost is lower; 2) several types of IF would be induced, and thus the mixture may be more effective than any *one* type; and 3) higher titers of IF may be achieved systemically and locally with inducers than with exogenous IF. Unfortunately, these clinically tested inducers, such as pyran copolymer and poly I:C, have been highly toxic. Some have attempted to decrease the toxicity of materials, such as poly I:C, by altering it to make it more susceptible to enzymes which break down RNA, with the hope that the inducer will last long enough for IF synthesis to begin but not long enough to produce toxicity. Others have prolonged the lifetime of poly I:C by complexing it with polylysine. The complex has reportedly been a potent IF inducer in man and nonhuman primates and has protected monkeys from several virus infections. A potentially persistent problem with the inducers, but not with the IF, is the development of hyporesponsiveness or tolerance to repeated induction or allergy to them that will interfere with effective repeated administration for long periods.

Accelerated research in IF in culture and in animals has documented their effects on viruses and viral infections, immunity, and several types of cancer. The mechanisms for the various activities require further investigation. The results of studies in man on the effects of IF on several viral infections associated with the immunosuppressed patient and against several neoplasms are encouraging. Although the anticancer effects of HuLeIF are preliminary, mostly unpublished, and only modest, they do suggest therapeutic potential. Whereas some of the anticancer effects observed in man with expensive HuLeIF may represent only as much anticancer activity as that observed with inexpensive cytotoxic chemotherapeutic agents, the fact that the

therapeutic results with HuLeIF were obtained with grossly impure material administered on what may be a suboptimal regimen encourages one to believe that, when more purified material is administered in optimal doses and regimens, far greater therapeutic effects will be observed.

Accordingly, for the BRM Program, the major obstacle to progress in this area, i.e., the availability of material, must be overcome. In in vitro and in animal models the mechanisms of IF effects on viruses, tumors, and immunological reactions should be determined; and the human IF should be tested in well-designed phase I and II studies in appropriate patients. As an editorial in *The Lancet,* June 2, 1979, stated: "Prediction in medical science is a foolish exercise, but it seems likely that interferon is going to prove useful in the management of malignant disease, though much more work is needed to find out how and when it should be used."

Thymic Hormones and Factors

Thymosin was the first extract from thymic tissue found capable of replacing the thymus in the development and maturation of T-cell-dependent reactions in animals without a thymus. Concurrent with the extraordinary increase in knowledge of basic cellular immunology, lymphoid cell heterogenicity, and cellular interactions, and with the development of a large array of sophisticated assays for specific immune functions in vitro and in vivo, thymic hormones and factors have been developed and shown to regulate or influence selectively or preferentially a given component of an immune response.

The hormones and factors are at various stages of purification, characterization, sequencing, and synthesis. Details of their chemistry and biology are presented in Section IV. A biologically active fraction from calf thymus termed "thymosin" was fractionated and purified to yield thymosin fraction 5, the material which has received the most attention and the greatest use in clinical trials. It consists of a number of polypeptides, one of which, designated thymosin alpha 1, has been sequenced and synthesized. Other polypeptides such as thymosin alpha 5, thymosin alpha 7, polypeptide beta 1, thymosin beta 3, and thymosin beta 4 have all been isolated and studied for biological activity.

The components of the immune response influenced by thymosin and/or its polypeptides in normal and thymectomized animals as analyzed in vitro and in vivo are discussed in Section IV. Functional assays, such as for cytotoxic, helper, and suppressor cell activity, cell surface markers, such as for Lyt phenotypes, and measurements of terminal nucleotidyl transferase have suggested differential effects of the various factors on distinct stages of T-cell maturation.

Other thymic hormones, such as thymopoietin and the FTS (both of which have been sequenced and synthesized), the THF, TFX, homeostatic thymic hormone, lymphocytopoietic substances (TP_1 and TP_2), thymic epithelial supernatant, thymus-dependent human serum factor of Staldi, and several other factors are at various stages of chemical and biological characterization. The influence of some of them on immunologic reactivity has also been documented.

Although the major thymic hormones affect responsiveness to various antigens and, possibly, to animal tumors, there is still little evidence that this effect may be used to the host's benefit in the therapy of an established tumor. However, it is conceivable that, with sufficient knowledge, our use of these hormones therapeutically may be possible eventually in normal models to enhance specifically, selectively, or preferentially the particular component of immunologic reactivity involved in tumor destruction.

Clinically, several thymic hormones, such as fraction 5, THF, and TFX have been shown to enhance the percentage of T-cells in the peripheral blood of patients, either when their blood was incubated with the thymic material or when the thymic hormones were administered to the patients. The largest number of patients to be treated with thymic hormones have been children with primary immunodeficiency diseases with syndrome attributable to failure of maturation of the normal thymus gland. A significant number of the patients responded with increased T-cell number and, often, T-cell function (as assayed in vitro) and, less frequently but significantly, with improvement in their overall clinical condition. Patient cells which responded to in vitro incubation with thymosin also responded to in vivo administration of thymosin. Approximately 10% of the patients exhibited mild side effects. Overall, although fraction 5 has been beneficial to some patients with DiGeorge syndrome, Wiskott-Aldrich syndrome, and chronic mucocutaneous candidiasis, evaluation of the therapeutic effects of thymic hormones in primary immunodeficiency stages is difficult because the number of patients treated remains small, the immunodeficiency states were heterogenous in type, the diseases varied in severity and clinical course, and the laboratory and clinical results obtained also varied greatly. More patients will have to be treated under more constant conditions with more readily available and well-defined thymosins before a reproducible benefit can be established.

The potential use of thymic hormones in cancer therapy must be based on the following assumptions: 1) Patients with certain types of cancer have certain identifiable immunologic defects associated with a poor prognosis; 2) thymosins when given in vivo will correct the immunological defects; and 3) correction of such defects, whether due to cancer or treatment for cancer, will improve the prognosis and survival of the patients. These assumptions are in the process of being tested. Various immunological defects and aberrations have been reported in patients with different types of cancer and in those undergoing conventional anticancer therapy. We need additional research to discover the reversibility of such defects by thymic hormones used in vitro and in vivo. Finally, well-designed studies are needed so that we can determine whether thymic hormones will improve the prognoses of patients with documented immunological abnormalities associated with neoplastic disease when used alone or as an adjunct to other modalities of therapy. However, such studies must be preceded by phase I trials with appropriate immunological monitoring.

A number of phase I–II studies are being performed with fraction 5 and thymosin alpha 1 on patients with head and neck carcinoma, oat cell carcinoma of the lung, and non-oat cell carcinoma of the lung. One phase II prospectively controlled trial has been reported on 55 patients with nonresectable oat cell carcinoma of the lung who were randomized to receive intensive combination chemotherapy with or without fraction 5. The median survival time of patients treated with chemotherapy plus thymosin versus no thymosin was 450 and 240 days, respectively. The increased survival correlated well with the initially low percentage of T-cells and low levels of alpha 2 HS glycoproteins. The results, though encouraging, are limited by the unusually short survival of the chemotherapy controls and by the small number of patients involved.

The thymosins are exciting and important agents with potential for increasing immunological reactivity to various antigens, and possibly, to TAA. Encouraging data suggest that thymosins can reverse or correct certain types of immunological defects in primary immunodeficiency states and perhaps in some cancer patients. The results of thymic hormone therapy for cancer are, at best, suggestive and preliminary. However, the basis for optimism about the potential of thymosins in cancer therapy is not the demonstrated anticancer activity in animals or man but the hope that patients with cancer will have specific defects in immunological reactivity due to their disease and/or therapy for their disease, that these defects will be correctable by thymosins with regimens and schedules to be derived from phase I studies, and that such correction will be associated with both improved anticancer therapy and survival. On that basis, support is justified for the characterization, purification, analysis, and use of various thymosins and for studies of their effects in appropriate in vitro and in vivo animal models and in man.

Augmenting and Immunomodulating Agents

Nonspecific immunotherapy in the form of agents that modify, restore, or augment immunity has received much attention during the past 20 years and with mixed results. The rationale is that the host's immunological response to putative TAA would increase and thus the tumor would be destroyed. The agents which have been most frequently studied have been microbial adjuvants, especially BCG, *Corynebacterium parvum,* and MER of BCG. Thus numerous investigators have examined effects of BCG on immunological reactivity and on cancer in animals and man. In recent years, our knowledge of the complexity of immunological responses and the ability to monitor specific components of such reactivity have increased dramatically. Concurrently, knowledge of the biology and clinical course of malignant disease has increased, conventional modes of therapy for many types of cancers have become more effective, and the requirements and characteristics of a good clinical therapy trial have become better recognized. These developments have led to a reevaluation of results obtained with immunoaugmenting agents that has revealed their deficiencies and identified some of the problems which must be resolved for adequate evaluation of the utility of this approach for cancer immunotherapy.

As the prototype agent, BCG has been exhaustively studied in animals for effects on immunity to tumor and non-tumor-related antigens. This agent has been reported to exert various effects on virtually every measurable immunological function, partly depending on the antigen, the particular immune reaction involved, and the dose and regimen of the BCG given. However, its principal effects are to stimulate or activate macrophages and cytotoxic lymphocytes and induce the production of IF.

The results of tumor therapy have also been extremely varied. Generally, BCG has been effective prophylactically in preventing tumor growth and therapeutically when given at the tumor site. However, with rare exceptions, BCG has been ineffective when administered systemically after a tumor has been established, and, in several models, even an established tumor has been susceptible to BCG given as an adjunct to chemotherapy. Moreover, BCG has been capable of reversing chemotherapy-induced immunosuppression and of possibly accelerating marrow recovery after chemotherapy. *Enhanced* tumor growth associated with BCG therapy has also been reported.

By analogy to animal studies, BCG has been the subject of clinical trials for various neoplasms with variable, unclear, or controversial results. The BCG has been administered at the site of the tumor or systemically. Locally injected BCG has unquestionably been associated with regression of most injected melanoma skin nodules in immunocompetent patients and of noninjected lesions in about 10–20% of them. Intravesical instillation of BCG has also been significantly effective against superficial bladder carcinoma. Furthermore, intrapleural BCG has been associated with a significant reduction in recurrence of stage I lung carcinoma; this is an important finding which must be confirmed.

The use of systemic BCG either alone or in conjunction with other modalities of therapy for disseminated disease or as adjuvant therapy to prevent recurrence has yielded some results which are modestly positive, many that are clearly negative, and many which are not sufficiently interpretable due to poor study design or to inadequate numbers of patients. No 2 studies have been performed identically. Indeed, some studies can no longer be performed identically because of progress in the use of other modalities of therapy. For example, Mathé's impressive chemoimmunotherapy regimen for ALL in children cannot be retested because the chemotherapy component is now unacceptable and far more effective chemotherapy regimens are currently available. However, once the chemotherapy is changed, the *immunotherapy* component may not necessarily exert the same detectable effect. Consequently, the failure of scientists to detect significant benefit with BCG in childhood ALL in a number of subsequent well-defined studies cannot be interpreted as an indication that BCG is ineffective or that Mathé's results were invalid because the chemotherapy regimens were different. A greater problem was posed by poorly designed studies which yielded encouraging results and led investigators to draw premature, overoptimistic, and overenthusiastic conclusions that, in turn, increased the expectations of the public and results which, when not confirmed by other research or by longer follow-up, led to widespread disappointment and

caused a credibility gap among clinician-scientists and the public.

The major problems with BCG clinical trials have been: 1) The BCG used was crude, variable in the ratio of viable-to-nonviable organisms, obtained from different sources and administered in empirically derived and changing doses and regimens; 2) the variable regimens used were derived without identifiable tests which would predict or correlate with a clinical immunotherapeutic response; and 3) the design of clinical therapy trials has too often been faulty and conclusions drawn prematurely and overoptimistically. Far less data have been generated in vitro and in vivo with *C. parvum* and MER which, generally, have been less effective.

To eliminate the problem of unreproducible dosage presented by crude material, investigators should progress to a second generation of immunoaugmenting agents which are well characterized, purified, and ultimately synthesized. Preclinical studies of their effects on components of immunological responsiveness and on tumors in appropriate animal models should identify those agents which are ready for clinical phase I studies of toxicity, with concurrent monitoring of effects on immunological responses. Clearly, the design of phase II clinical trials must be based on rigorous criteria of preclinical activity and clinical monitoring.

Of the agents that are appropriate candidates for such testing, one specifically proposed in the BRM Program initially is one of several defined fractions of pyran largely because these fractions, designated MVE-1 through 5, are active in several tumor models. They appear to have limited toxicity compared with the crude pyran copolymers, which have been reported to have many effects on immunological reactions in animal models but, which, in a human phase I study, had been used in excessive dosages and were too toxic.

Immunoaugmenting agents were investigated for a long time with variable results. Their effects on immunological reactivity are becoming better defined, and their antitumor effects in animal models, though modest, are well established. Their therapeutic effects in man have been mixed, due partly to variations in the empirically derived doses and regimens of the crude materials and to poor designs of clinical trials. Nevertheless, the basic rationale for the use of immunoaugmenting agents in cancer therapy remains valid, and preliminary results of several recent well-designed clinical trials are sufficiently encouraging to justify use of well-defined and characterized and/or purified agents in careful preclinical and clinical trials with appropriate assessment of clinical results.

At times, BCG and other agents which normally *augment* immunity have, in fact, suppressed it. With increased understanding of the interactions between lymphocyte subsets, it is now apparent that the end effect, i.e., augmentation or suppression of such agents, may be influenced or determined by the immunological status of the host being treated and the balance between helper and suppressed cells.

Numerous immunomodulating agents of this kind are becoming available and are being studied now, notably, indomethacin and cimetidine. Although levamisole has been beneficial as adjunctive therapy of patients with breast and lung cancers and multiple myeloma, conflicting results have also been reported. Thus its clinical effectiveness has not, in fact, been definitively established.

Agents which modulate immunological reactivity through regulation of prostaglandin synthesis by macrophages or tumors, best represented by indomethacin, have yet to receive the investigative attention they deserve. Similarly, agents which modulate immunity by blocking H_2 receptors on suppressor cells must be studied intensively in vitro and in animal models, and, if shown to deplete suppressor cells and benefit the animals, they should be tested in man. Cimetidine and metiamide represent that group of interesting and relatively nontoxic agents worthy of study.

Human Cancer Antigens

Attempts at modification of the immune response to human cancer are based on the assumption that cancer cells can be distinguished from their normal counterparts by the presence of distinctive cell surface antigens. Although the impression that TSA have been demonstrated in many types of human cancers is generally accepted, the existence of such antigens must still be considered uncertain. Specificity is the critical issue, and the specificity of serological or cell-mediated immune reactions is defined much easier in the mouse than in man. In the absence of the advantages offered by the availability of inbred mouse strains, the human cancer serologist is still attempting to develop approaches that can help him cope with the issue of specificity.

Most of the current knowledge of cell surface antigens of human cancers is derived from studies that require heteroimmune or allogeneic sera and lymphoid cells. However, apparently TSA defined by heterologous sera have always been, on more extended analysis, characteristic of normal cells at some stage of differentiation. Tumor antigens detected by reactions with human sera fall into several categories. Some are alloantigens, particularly products of the HLA complex and *ABO* locus. Another category of antigens is related to the EBV. The difficulty in one's interpretation of results of studies involving allogeneic sera is the unknown participation of alloantigens in the reactions observed. It is for this reason that most surveys for humoral immunity or CMI to cell surface antigens of human cancer cells have not permitted distinction of tumor-specific reactions from those directed to other categories of antigens. If we restrict the analysis to autologous combinations of serum and tumor cells, some of the difficulties involved in determining whether cancer patients recognize TSA on the surface of their cancer cells are eliminated. This approach, referred to as autologous typing, has been applied initially to the study of cell surface antigens of malignant melanoma, acute leukemia, astrocytoma, and renal cancer.

Three classes of human cancer cell surface antigens can be defined by autologous antibody. Class I antigens are individually distinct antigens, showing an absolute restriction to autologous cancer cells. Class 2 antigens are shared by some allogeneic tumors. Class 3 antigens are expressed on a wide range of normal and malignant cells of human and animal origin.

The finding that some patients have antibodies to cell surface antigens of their tumor cells raises the following questions: 1) Why is it that only few patients have antibody to Class 1 or 2 antigens? 2) Does the antibody titer remain constant or change with time? 3) Is a change in antibody titer detected by one assay paralleled by a similar change measured by another assay? 4) Do different assays detect antibodies of the same specificity? 5) Does the specificity of antibodies in the serum of a given patient change with time? 6) Do cell lines derived from different metastases of the same patient express the same Class 1 or 2 antigens? Only rudimentary information regarding these questions is available at the present time.

The development of the hybridoma methodolgy has added a new dimension to the serological analysis of human cancer cells. Monoclonal antibodies, produced by the hybrid progeny of a myeloma cell and a normal immunoglobulin-secreting B-cell, are likely to replace for many purposes conventionally prepared antisera and thus allow the preparation of highly specific reagents in large quantity. Several technical problems remain to be resolved, such as improvement of selection methods for isolating the desired clone and reducing clonal instability due to chromosomal loss. However, application of the methodology in its present form has already produced monoclonal antibodies to histocompatibility and differentiation antigens of man, mouse, and rat.

In the cancer field, several groups have produced hybridomas secreting monoclonal antibodies to human cancer cell surface antigens by immunizing mice with human cancer cells and fusing their spleen cells with mouse myeloma cell lines. Various systems of human cancer cell surface antigens have been defined with monoclonal antibodies, each with a characteristic cellular distribution, and investigators' current efforts are directed at characterizing these antigens in biochemical terms. Some of the antigens show a highly restricted distribution, but none of the antibodies developed so far recognize cancer-specific cell surface antigens.

Mouse monoclonal antibodies can only tell us, of course, what the murine immune system recognizes on human cancer cells. As human myelomas are now becoming available for fusion with human lymphocytes, it may be possible for us to produce human monoclonal antibodies and define more precisely the cancer cell surface antigens that can be detected by the human immune system. As a consequence of the intense activity in this area, we can look forward to a comprehensive picture of the surface antigenic structure of human cancer cells. This work may eventually lead to new classifications of tumors, once correlations of certain clinical features with the presence or absence of cell surface antigens detected by these antibodies have been established. Apart from being powerful analytical probes, monoclonal antibodies may also have important applications in diagnosis and therapy.

T-cells for Cancer Therapy

Lymphocyte transfer as a form of adoptive therapy for cancer has been studied since the original demonstration that rejection of antigenic tumor in animal models was mediated largely by immune cells reactive to TAA. Some of the assumptions on which the approach is based, about the existence of TAA, the nature of the effector cell, the host–tumor relationship, etc., have been validated in some animal models, whereas their validity in man is still being tested.

Approximately 10 to 15 years ago, animal models for adoptive therapy were developed. Therapeutic use of lymphocytes was also attempted in *patients* with cancer but with inconclusive results. A number of developments which make it more likely that adoptive therapy will eventually have a role in the treatment of cancer in man have sparked a resurgence of interest in this approach. These include the following: 1) More animal models have been reported in which immune T-cells can eradicate established tumor; 2) T-cells can now be primarily or secondarily sensitized to TAA in vitro; 3) sensitized cells can be maintained in large numbers for long periods in vitro in the presence of IL-2; 4) subsets of animal and human lymphocytes are being identified and cloned, and their interactions in the immunological network are better understood; 5) allogeneic cells, which are known to have an antitumor effect in animals, have been reported to exert an antileukemic effect in man.

The clinical potential of adoptive therapy can only be assessed from studies in animal models which, though not obviously analogous to a clinical situation, can be used in identification of problems to be resolved before clinical application. To date, the most extensive research has been performed in totally syngeneic models with cells immune to strongly antigenic tumors. Although such cells can eradicate tumor when infused shortly before or after tumor inoculation, their efficacy is limited by a large tumor burden and by the development of host suppressor factors, especially suppressor cells. Accordingly, a number of models have been developed in which cells immune to TAA are effective against advanced tumors when used as an adjunct to other modalities, such as cytotoxic antitumor chemotherapy and/or radiation, modalities which can decrease both the tumor burden and the generation of suppressor cells and thereby promote the efficacy of the infused donor cells.

In investigations of the mechanism of adoptive immunotherapy, the critical donor effector cell in vivo in every therapy model studied has been an immune T-cell. Moreover, the particular subset of donor T-cells critical for therapy has been identified in 2 models as a T-cell which is *not* cytolytic to tumor in vitro but probably amplifies the cytotoxic T-lymphocyte response in vivo. The results suggest that, for effective therapy, a donor amplifier T-cell may be required for induction of a host-derived effector mechanism in vivo and for highlighting the important and potentially modifiable role of the host in adoptive therapy.

Effective therapy requires tumor-immune effector cells. We can now generate these by sensitizing lymphocytes in vitro. Primary sensitization to tumor in vitro makes normal cells immune without exposing the normal donor to tumor. Secondary sensitization to tumor in vitro can generate more effector cells with enhanced antitumor reactivity. Furthermore, it represents a way suppressor cells or other mechanisms can be bypassed that normally limit the generation and/or expression of antitumor

effector cells in a tumor-bearing host. In animal models, cells sensitized in vitro, especially those cells primed in vivo and secondarily sensitized in vitro, are therapeutically effective against established tumor. One problem encountered during sensitization in vitro is the concurrent generation of suppressor cells which can demonstrably interfere with the therapeutic efficacy of effector cells. Approaches to preferential generation of the effector cells must be developed.

To be therapeutically effective against advanced tumor, the immune cells must be given in large numbers. This prerequisite can now be met when immune lymphocytes are maintained in long-term culture with IL-2. Such cells maintain their in vitro cytolytic function and, in one model, can be immunospecifically therapeutically effective in vivo. Numerous investigators have cloned T-cell subsets and studied their therapeutic efficacy.

Syngeneic models are useful for the study of mechanisms of adoptive therapy without the complications posed by donor–host histoincompatibilities. Although the only grossly analogous clinical setting is one in which the lymphocyte donor and tumor-bearing recipient are genetically identical twins, the principles derived from syngeneic animal models may be widely applicable to patients whose autologous lymphocytes might be considered as primed to tumor. Such cells can be resensitized or otherwise manipulated in vitro, so whatever immunosuppressor factors might be operative in vivo can be bypassed and then reinfused.

Studies with allogeneic lymphocytes are far more complex and less frequently successful. Allogeneic lymphocytes alone are rarely therapeutically effective against established tumors but can be so when given as an adjunct to radiation and especially to chemotherapy. Results from the few successful models suggest that, for maximal effectiveness, the cells should be immune to tumor and should persist and proliferate in the host for some time. These prerequisites emphasize the need for approaches by which sensitized cells in the presence of histoincompatibility can be generated. Moreover, the apparent need for persistence of donor cells in the host requires that the noncellular modality of antitumor therapy be immunosuppressive so as to prevent rapid rejection of donor lymphocytes by the host, which, in turn, leads to the central problem of potentially fatal GVH disease. A number of approaches are being investigated for prevention of GVH disease. However, control rather than prevention may be preferable because the GVH disease can exert an effect against some animal tumors, even when the donor lymphocytes are not immune to host or to tumor.

Thus several principles of adoptive immunotherapy have been derived from studies in syngeneic and allogeneic animal models. Obviously, more must be learned about the mechanism of adoptive immunotherapy, so that we can better appreciate the prerequisites for efficacy and identify the steps amenable to modification. We must apply these principles to the development of additional animal models in which the tumors are less immunogenic and of more recent origin, in the primary as well as secondary host, so as to mimic more closely the presumed clinical setting.

Many cancer patients have been treated empirically with lymphocytes with inconclusive results. However, the conditions were not consistent with the prerequisites derived from animal models. Thus no valid adoptive immunotherapy study analogous to the studies performed in animals has been reported in man. The only approach which might represent adoptive immunotherapy is that which involves the treatment of patients with hematologic neoplasms with supralethal doses of chemoradiotherapy and bone marrow transplantation from autologous, syngeneic, or allogeneic sources. That normal syngeneic marrow transplantation has been associated with an immunologically mediated antileukemic effect has not been substantiated. However, there is excellent circumstantial evidence for an immunotherapeutic effect by allogeneic marrow in man as reflected by a strong association between the occurrence of significant GVH disease and a decrease in leukemic relapse after transplantation.

For eventual clinical application of adoptive immunotherapy, the issues raised in animal models should be investigated by analogous studies in man. The existence of antigens on human tumor (unique or shared by other tumors and alloantigens or differentiation antigens) must be documented so the appropriate immunogen for in vitro sensitization to tumor can be identified. We should continue efforts to generate and maintain human effector cells in long-term culture, clone them, and test their interactions and their antitumor activity in vitro. The antitumor reactivity of lymphocytes from tumor patients should be determined as should the effect of various in vitro manipulations on that reactivity. Finally, approaches to the control of clinical GVH diseases are already being pursued.

In conclusion, during the past decade, animal models for effective therapy have been developed, some of the apparent prerequisites for therapeutic efficacy in the models have been determined, and the problems those prerequisites pose for potential clinical extrapolation have been identified. Although no directly analogous approach has been shown to be effective in man, the reported antileukemic effect of allogeneic marrow in man, as in animal models, is most encouraging.

Several additional parallel developments in man and animals markedly increased the likelihood that cell transfer will have a role in clinical cancer therapy. The existence of TAA has been demonstrated in many animal models. The problem in man is far more complex but should be more soluble with the aid of monoclonal antibodies. Cytotoxic lymphocytes can be generated, maintained, and cloned with some demonstrated specific effector function in animal models, the interactions of subsets of cells, and the effector cells being progressively better understood. Similarly, subsets of lymphocytes in man with different functions, such as cytotoxicity, and helper and amplifier functions are being identified by monoclonal antibodies. Human lymphocytes cytotoxic to tumor cells can also be generated and maintained. Moreover, we can now potentially manipulate the subsets so as to eliminate reactive T-cells and prevent GVH disease, or eliminate suppressor cells and increase host reactivity to the tumor and/or allow the immunotherapeutic efficacy of infused immune donor cells. Continued exploration of these areas in animal models and, when appropriate, in man, will undoubtedly

better define the potential role of adoptive cell transfer as a specific component of cancer therapy.

Tumor-preventive Agents

Potential prevention of malignancy must be based on an understanding of the mechanisms underlying chemical carcinogenesis. Such carcinogenesis is generally divided into 5 major processes, i.e., exposure to the carcinogenic agent, metabolic activation of carcinogens to proximal carcinogens, reaction of the molecules with target sites within the cell, fixation of chemical damage as biological damage, and the progression and expression of the initiated cells. The latter process is accelerated by cocarcinogens called tumor promoters which themselves are not carcinogens but which potentiate the action of the carcinogen when administered after but not before that agent. Although the initiator causes a permanent change in the cell, the tumor promoter to have any effect must be applied repeatedly; it also causes changes which are not permanent. Models for tumor promoters have been developed, and the possible efficacy of agents that might interfere with promotion and therefore carcinogenesis has been tested.

Natural synthetic compounds related to and including vitamin A (we refer to all as retinoids) can prevent and occasionally reverse tumor progression and can antagonize the actions of experimental promoters. In studies of in vivo models of organ-specific chemical carcinogenesis in animals, retinoids delayed the development of chemically induced cancers of the breast, bladder, skin, colon, and respiratory tract. The studies involved chronic administration of retinoids after exposure to highly potent carcinogens given at concentrations large enough to cause cancer in most of the exposed animals. Such therapy might be more effective in man because lower concentrations of carcinogens are involved. Similarly, work with organ cultures of the prostate and trachea has shown that retinoids can reverse chemically induced metaplasia, and in that in which cell cultures were used, especially transformable mouse fibroblast cell lines, not only can retinoids delay the development of neoplastically transformed cells exposed to chemical carcinogen but their effect is reversible. We want to emphasize that the retinoids have always been administered chronically for long periods, and their cessation was often associated with reemergence of malignant cells.

Studies with retinoids have also suggested that they can augment immunological reactivity of both B- and T-cell origin and, under certain conditions, can retard tumor growth when used alone or in conjunction with chemotherapeutic agents. However, neither the immunological effects, especially those reflecting tumor-specific reactivity, nor the antitumor therapeutic effects, most of which are assumed to reflect and be mediated by immunologic mechanisms, have been established definitely for the retinoids.

Among the epidemiological and therapeutic clinical studies investigators performed, those conducting epidemiology research suggested that a low vitamin A level was associated with a significantly higher risk of lung cancer in smokers and bladder cancer in smokers and coffee drinkers. On the other hand, therapeutic studies have shown that local therapy with retinoic acid was associated with a significant incidence of complete remission of actinic keratoses and of basal cell carcinomas, whereas oral retinoic acid caused a significant increase of complete and partial regression of papillomas of the urinary bladder. However, 13-*cis*-retinoic acid had no significant effect in preventing the recurrence of bladder cancer in a series of 80 patients with a history of recurrent, papillary, superficial transitional cell carcinomas.

Retinoids have interfered with carcinogenesis in vitro and in vivo and enhanced immunological responsiveness which, conceivably, may affect an established experimental tumor. Clinically, vitamin A deficiency is related to a higher risk for carcinogenesis, and 13-*cis*-retinoic acid may be effective against skin cancers and bladder papillomas. The results are sufficiently encouraging to warrant support for the synthesis and investigation of more potent and/or less toxic retinoids, for the development of an effective, rapid, and reproducible in vitro screening method for substances with high chemopreventive potential, and for additional clinical trials in patients who are at high risk for the development of primary cancer or whose primary tumor has been removed but who are at great risk for late recurrence. It is also conceivable that the retinoids may have a greater effect on enhanced immunological responsiveness than is believed and may be used in other therapeutic situations as immunoaugmenting agents.

Hybridomas in Diagnosis and Treatment of Cancer

Exploitation of the hybridization technique first described by Kohler and Milstein for producing long-lived clones of antibody- or factor-producing cells has recently begun in earnest for the diagnosis, localization, and therapy of cancer, as well as for the dissection of the immune response to tumor cells. Myeloma cells, usually derived from mice given ip injections of mineral oil, so-called MOPC tumor cells, have generally been the cells most often hyperimmunized with which sensitized lymphocytes from mice or rats have been fused. With continual improvements in technique, the clones derived from this fusion persisted in tissue culture indefinitely and permitted a stable line of specific antibody-producing cells to be utilized for immunological studies. Literally grams of antibody can be produced, particularly if the clones are injected into mice after their derivation in vitro. Moreover, one can select precisely the class and subclass (isotype) of antibody one desires and thus its avidity for the antigen(s) and the specificity of the antibody. The titer of antibodies exceeds that which one can achieve by hyperimmunizing animals alone without producing hybridomas from the hyperimmunized cells. Production of human–human hybridomas has also been possible in a few instances with selected lines of human myeloma cells as the immunoglobulin-producing basic cell to which sensitized human lymphocytes can be fused. Hybridomas of malignant T-cells with sensitized T-cells have also been produced that lead to T-cell-derived factors which can be studied for their effects on tumor cells or immunologically active cells.

The uses of monoclonal antibodies derived from the hybridomas include all those to which conventional anti-

sera have been put. The major difference between the newer reagents and antisera is the intrinsic exquisite specificity of monoclonal reagents. In fact, that specificity is also their principal drawback because many monoclonal antibodies recognize narrowly only one or two antigenic determinants (epitopes) rather than several. This latter fact makes possible an erroneous conclusion that many unique antigens are present when only one or a few are actually active there. Furthermore, the decision whether one should use monoclonal antibodies derived from mouse anti-human versus human anti-human reactivity requires awareness of the situation investigated, inasmuch as the former types are far broader in their recognition of differences on human cells than are the latter types. Diagnosis of a tumor through detection of antigens in the serum might require only that an antibody react with that antigen, even if it reacts with other substances not found in the bloodstream. Thus one might not need a highly specific monoclonal antibody at all under those circumstances. On the other hand, specificity is critical if one wants to localize a tumor cell in normal tissue, especially for targeted cytotoxic therapy.

A principal use envisaged for monoclonal antibodies is the recognition of neoantigens on tumor cells, which has been a most difficult problem with conventional serological methods. Already a host of human tumor cells have been noted to react with monoclonal antibodies, and it is likely that panels of such reagents can be used in the classification of subtypes of tumor cells within a class and in diagnosis of the presence of the tumor in frozen or fixed histological sections, as well as by reactivity with serum antigens shed by the tumor. No one monoclonal antibody is likely to be a "pan-tumor cell" reagent, even within a histological category of tumor. Production of targeted cytotoxic reagents that would kill the tumor cell in the tissues specifically without harming normal cells is another distinct possibility with monoclonal antibodies. The likelihood is that the antibodies would be useful, particularly for dispersed tumors, such as leukemias, and perhaps for lymphomas, and might well be effective against some solid tumors, provided that their blood supply was good throughout and the antibodies could penetrate the bulk of the tumor. Dissociation of the toxic material (e.g., radioisotope, ricin, microbial toxin) before it reaches the tumor is a major problem that must be faced before routine use of targeted cytotoxic reagents will be possible. Antibodies alone might be useful for certain tumors but would probably require considerable assistance from effector cells, intrinsic or administered, if experience in animals holds true.

Radiodiagnosis of tumors in situ more specifically than with present scintigraphic methods should also be greatly facilitated through the use of tagged monoclonal antibodies. Microscopic metastatic deposits of tumor cells in the viscera might be identified through the use of mixed monoclonal antibodies (for coverage of a range of antigenic specificities) tagged with a conventional radionuclide with more sensitivity than is now possible.

Dissection of the lymphoid cells responsible for rejecting tumor cells will be facilitated with monoclonal antibodies. Already one can separate different types of T-lymphocytes through their antigenic markers and can learn by subtraction which are critical in reactions against tumors in mice. The possible manipulation of human lymphocytes through monoclonal antibodies is attractive, especially antisuppressor cell therapy. The same approach can be taken for classification of lymphoid neoplasms, so that subtypes with uniform prognosis and response to therapy can be determined. Much of this has already been accomplished with conventional antisera, but the pace of the work and its fineness will be enhanced with monoclonal antibodies. Researchers have done little to classify and dissect myeloid cells responsible for rejection of tumors or for the myeloid tumors; these too will be attacked with monoclonal reagents.

Finally, suppressor substances produced by T-cells could be antagonized by our developing antibodies to purified suppressor factors from cloned T–T hybridomas. In this circumstance, one can envisage monoclonal antibodies made against monoclonal factors, perhaps the ultimate extension of these methods.

Lymphokines as Biological Response Modifiers

Recent advances in our understanding of immunological reactions make it apparent that lymphokines are important in the development of host immunity and may be useful in modifying the biological responses in patients with cancer or other diseases associated with immune dysfunction. Lymphokines are the intercellular molecules derived from lymphocytes involved in the communication and amplification that occur between reacting cell populations in an immune response. One of the earliest extracellular evidences of immune reactions is the presence of lymphokines secreted by cells reacting to antigenic stimuli. Lymphokines are involved throughout the response and are essential in the immune reaction itself, influencing cell migration patterns, cell division, cell activation, effector cell function, and even the suppression of the response. Understanding of immune reactions requires a careful dissection of the various lymphokines that have roles in the maturation, differentiation, and effector functions of involved cell populations. Such knowledge will be necessary for the development of mechanisms whereby the immune system can be influenced to participate in antitumor activities by direct intervention with lymphokine agents.

The area of lymphokine research is one of intense interest among immunologists and is one in which great strides forward are being made. They have described many lymphokine agents in the literature, and, internationally, have tried to initiate standardization of terminology and identification of lymphokines by biochemical properties as well as by biological activities. Lymphokines are presently being purified and characterized by numerous investigators. Several have been purified to apparent homogeneity, such as TCGF (IL-2) and are being sequenced. Others have defied efforts at isolation (such as MIF and MAF) and are still available only in a partially purified form. Some lymphokines induce cell proliferation and lead to the generation of specific clones of cells wholly dependent on that particular factor for continued proliferation and survival. Other factors induce functional differentiation of effector cells capable of mediation of specific

immunological functions (such as cytotoxicity), whereas still others (such as IF) may serve to regulate immune responses. Other lymphokines (such as lymphotoxin) may be the effector molecules that cause certain portions of the immune response to occur by their effects on the local physiology of the site of reaction or the agent responsible for effecting the immune response by direct lytic activity on cells. Some lymphokines (such as T-cell replacing factor) can replace their cellular source in an immune reaction, making possible the induction of immunological reactions in the absence of specific subpopulations of cells. Such dissection of the immune response is what is needed for us to develop cellular models in which definitive interactions between lymphokines and cells can be detected, studied, and quantitated. Progress in understanding the function of each lymphokine in the immune response will lead to the development of our ability to manipulate immune responses extrinsically to the benefit of the patient by initiating or eliminating specific components of the immune reaction as clinically desirable.

Can such exciting possibilities be considered in therapy regimens for cancer patients? The fact that many of the cells and mechanisms involved in the defense against tumors are also directly involved with lymphokine production or utilization gives ample justification for the evaluation of such use of lymphokines. Initial trials are already underway in Europe and the United States in phase I studies with crude lymphokine-containing material derived from a human lymphoblastoid cell source (RPMI-1788 cells). These trials are supplying information necessary for the development of more sophisticated protocols with purified lymphokines of higher potency.

Many questions need to be answered before the use of lymphokines as a treatment modality can be critically evaluated. These questions include: 1) the best method of administration, 2) the biological half-life of injected lymphokines, 3) the effects of nonlymphokine components on lymphokine responses, and 4) the systemic toxicity of lymphokines. Because results with IL-2 indicate that the biological half-life of lymphokines may be short, the effects of lymphokines may be strictly mediated in vivo through the cell populations acted upon by these agents. Pretreatment with monokines or thymic hormones may greatly influence the size of the cell population that will respond to the lymphokine treatment. As lymphokines are further purified, protocols for sequential administration for desired effects must be developed.

The exact role and mechanism of action of various lymphokines will probably be best evaluated in animal models initially. Scientists have launched a great effort to develop the necessary reagents and methodologies to dissect different populations of lymphocytes and macrophages for experimental protocols. Experimentation on the segregation and reassembly of immune responses with cell subpopulations or lymphokines is currently evident in the literature. This approach is essential for determination of the activity of various cells for lymphokine production and the effects of lymphokines on cell subpopulations during immune reactions.

Although recent progress in research on lymphokines has given us a basis for optimism in the feasibility of initiating and controlling immune responses or in correcting defects in the immune system by supplying missing or defective lymphokines, it must be remembered that the immune system is a "two-edged sword" and has profound capabilities for detrimental effects as well. A nonregulated lymphokine-induced immune response could have serious adverse effects on patients. Results to date have suggested that lymphokines may have selective effects on endocrinological function (such as inducing elevated corticosterone levels in blood) and on WBC compartments (such as inducing transient neutrophil leukocytosis and lymphopenia) which deserve careful attention.

Progress in this area of research will be greatly enhanced by 1) the identification of the mechanism by which lymphokines mediate their activity on cell populations and by which their production is regulated; 2) standardization of specific lymphokine assays with activity being measured in reference to an international standard; 3) the development of large-scale sources of lymphokines (preferably of human origin) from either cell lines, hybridomas, or recombinant DNA technology; 4) the purification of lymphokines to homogeneity and characterization of their biochemical properties; and 5) the production of monoclonal antibodies against specific lymphokines.

Our ability to use lymphokines effectively to intervene in immunological functions in patients by selectively amplifying or suppressing components of the immune response will ultimately depend on our ability to define the molecular and cellular mechanisms by which lymphokines must emanate from a concentrated effort to define the specific mechanisms of differentiation and maturation that are induced by these potent BRM.

Maturation (Differentiation) Factors in Cancer

The apparent maturation block associated with neoplastic transformation in a variety of cell systems can be a reversible phenomenon. Terminal (postmitotic) differentiation can be induced by various physiological and pharmacological factors. When investigated, the proliferation and/or differentiation of renewing cell populations was shown to be controlled by specific growth regulatory factors, generally of a glycoprotein or polypeptide nature. Contrary to earlier beliefs, most transformed cells retain some dependence on growth regulatory factors of exogenous or endogenous origin and thus manipulation of production or action or growth and maturation factors in cancer therapy.

In some cell systems, growth factors induce proliferation of target cells without inducing further differentiation. Epidermal-, fibroblast-, and platelet-derived growth factors are well-characterized molecules which can be categorized as mitogenic proteins for their respective target tissues. Inappropriate production of such factors by transformed cells or the production of functionally similar molecules following viral transformation (e.g., SGF) confers a proliferative advantage on the transformed cell. In these instances, we should base our therapeutic strategy on blocking the synthesis and release of the growth factor on which tumor proliferation depends, or on modulating or blocking receptors for growth factors, possibly using

synthetic peptide antagonists or monoclonal antibodies against the growth factor or its receptor. An alternative therapeutic strategy for growth factor-dependent tumors could be the use of exogenous growth factors for induction of cell cycle synchronization of tumor cells in conjunction with cycle-specific chemotherapeutic agents.

One of the first physiologically relevant agents shown to induce terminal differentiation of malignant cells was NGF, which is a small basic protein of 13,500 daltons or a noncovalently linked dimeric form. Inhibition of growth with induction of terminal differentiation can be seen with many neuronal tumors derived from neurectoderm and exposed to NGF. Thus NGF is of therapeutic significance in neuroblastoma, retinoblastoma, medulloblastoma, and pheochromocytoma. Tumors of the latter type can exhibit a proliferative response to EGF but this can be overridden by NGF by a mechanism that involves the ability of the latter factor to limit the synthesis of receptors for the stimulatory EGF.

Further research into this mechanism is warranted because it may provide a common mechanism by which maturation factors act, i.e., by inhibiting synthesis of receptors for growth-stimulatory (mitogenic) factors.

Hematopoietic growth regulatory factors are characterized to varying degrees. The biological properties of lymphoid growth factors, such as IL-1, 2, 3, are discussed in detail elsewhere in this Report. Malignant T-cell populations can depend on or produce IL-2 and thus could be amenable to therapies based on manipulation of that growth factor. Considerable attention has been paid to hematopoietic CSF (so-called because they are assayed in in vitro clonogenic assays). The best characterized of these factors are the G- and M-CSF required for the proliferation *and* differentiation of myeloid progenitor cells in bone marrow. The growth factors are required continuously throughout the maturation sequence and can even modulate protein and RNA synthesis in mature polymorphs and macrophages. A glycoprotein specific for monocyte–macrophage development, termed "M-CSF or CSF-1," has been isolated from various species, including man, and, though it shows some species specificity, the factor consists of 2 polypeptide chains, is heavily glycosylated, and has a mol wt in the range of 40,000–86,000. A GM–CSF of 23,000 has been purified from murine sources and is equally effective in stimulating granulocyte and macrophage development, whereas a less well characterized 35,000-dalton G-CSF stimulates only neutrophil maturation. In the context of malignant hematopoiesis, all primary human myeloid leukemias, both acute and chronic, depend on CSF for their in vitro proliferation, and the bulk of CSF species appear unable to induce terminal differentiation of leukemia cells. The recognition of heterogeneity and species specificity of CSF plus the "dependent" nature of human myeloid leukemia indicate a priority for improved purification and production of large quantities of these factors. A number of human cell lines have been identified as constitutive producers of CSF and would provide a suitable source for large-scale production. The CSF may be utilized to cycle-activate G_0 leukemia stem cells in conjunction with S phase-specific chemotherapy. The factors may also be valuable in augmenting myelopoiesis in patients receiving intensive chemotherapy and/or radiotherapy for nonhematopoietic cancers, and both GM- and M-CSF may be beneficial in activating macrophages and enhancing their capacity to mediate nonspecific tumor cell killing.

Much attention has been paid to various leukemia cell line models that can be induced to terminal differentiation. The most extensively studied have been Friend erythroleukemia and the mouse myeloid leukemia cell lines M-1 and WEHI-3 which can be induced to granulocyte and/or macrophage differentiation by compounds as diverse as DMSO, dactinomycin, endotoxin, phorbol esters, dexamethasone, and various protein inducers. The human promyelocytic leukemia cell line HL-60 can likewise be induced to terminal differentiation to granulocytes by exposure to polar compounds or retinoids and to macrophages after treatment with phorbol esters. The existence of physiological inducers of myeloid leukemia cell differentiation has been documented in various systems; the most consistent is the observation that serum collected 3–6 hours following endotoxin administration in man or mouse could induce terminal differentiation of myeloid leukemia cell lines.

Biochemical characterization of postendotoxin serum has shown that the DF was a 28,000-dalton protein and could be separated from the bulk of serum CSF but coeluted with a minor species of CSF that stimulated only granulocyte colony formation. The cellular origin of endotoxin-induced differentiation protein (GM–DF or MGI-2) is unclear, but macrophages are likely candidates and, in this context, evidence is pointing to the capacity of myeloid leukemia cells to produce their differentiation-inducing protein. Indeed, differentiation-inducing agents, such as endotoxin, phorbol esters, and glucocorticoids appear to act indirectly and promote differentiation by inducing leukemia cells to produce their DF.

Evidence for the inducibility of terminal maturation of malignant cells is based on extensive use of a few tumor cell lines selected for their capacity to differentiate. The generality of this phenomenon requires further investigation in primary tumor culture systems. However, good evidence suggests that, even if terminal differentiation of malignant cells is not obtained, differentiation-inducing agents such as retinoids and GM–DF block self-renewal of malignant clones.

Synergism between BRM has been demonstrated, and, in the context of tumor cell differentiation, retinoids plus prostaglandin E or GM–DF plus low concentrations of dactinomycin are significantly more effective than each agent individually in promoting terminal leukemia cell differentiation. Indeed, leukemia cell lines selected for their inability to differentiate (D^- clones) can be induced to differentiate and lose in vivo tumors only when exposed to a combination of dactinomycin as a sensitizer and a differentiation inducer such as GM–DF or retinoic acid.

Phase I and II clinical trials of differentiation-inducing agents should be undertaken in cancer patients when in vitro evidence indicates the possibility of differentiation (i.e., germinal, neurectodermal, and hematopoietic tumors). Trials with combinations including conventional chemotherapeutic agents are also warranted, inasmuch as the efficacy of certain chemotherapeutic agents may reside

not so much in their ability to kill malignant cells selectively, but rather in their capacity to induce production of endogenous differentiation factors and/or to sensitize tumor cells to such endogenous influences.

Support is also justified for more extensive purification and large-scale production (with the use of recombinant DNA technology) of these maturation and proliferation factors with the prospect of clinical trials. Monoclonal antibodies should be developed against the factors that would allow RIA, and affinity purification and in vivo "immune ablation" studies to be undertaken. Reference preparations of highly purified growth and maturation factors should be established, standard criteria for units of biological activity agreed upon, and a formalized mechanism of exchange of factors between investigators established.

RECOMMENDATIONS FOR PROGRAM IMPLEMENTATION

General Description of Operation Phases

Because of the particular nature of the BRM Program, a substantial portion of its preclinical developmental aspects cannot be usefully structured within predetermined and well-defined schemes and is best outlined only in general terms with the purpose of stimulating needed investigator-initiated research. It is this research effort that represents the source of agents and ideas which are directly inserted into the more structured part of the BRM Program. For maximum efficiency of Program development and progress achievements, it is essential that both the investigator-initiated and the structured developmental parts be considered as closely interrelated components of the same BRM Program.

The more structured part of the Program would consist essentially of a Development Network which starts at the time a BRM is accepted for development toward clinical trial, based on information acquired elsewhere, and ends at the completion of phase III clinical trials.

The information acquired elsewhere would include preclinical data obtained on individual agents or on small sets of agents, in academic, industrial, or intramural NCI laboratories, and clinical data obtained outside the Program. For particular series of analogs within promising BRM groups, synthesis and/or preclinical screening may be performed under the aegis of the Program prior to acceptance into the Development Network. Also, for compounds derived from outside sources, evaluation in preclinical systems may be confirmed within the Program. Thus in this Report, a system of preclinical screening of BRM has been recommended. Because of the present availability of agents needing prompt development toward the clinical trials, suggestions about how the Development Network can best be implemented were formalized in some detail.

Inasmuch as agents being screened by the Division of Cancer Treatment for anticancer activity may also act as BRM, it seems important that a small signal system of selective tests be inserted into the Division's Chemotherapy Screening Program that would identify compounds with BRM activity. This small signal test system should be inserted between stages IIA and IIB in the chemotherapy linear array, and it could serve as a bridge toward the BRM Program.

Development Network

A schematic outline of this Network as proposed by the Subcommittee is shown in figure 1-1. (For actual Program implementation, *see* the Appendix prepared by Dr. Robert Oldham, BRM Program Director.) Often information on new candidate BRM is likely derived from a data base obtained outside the Program. Information is also expected to be derived through feedback from the investigator-initiated research part of the Program or from the Division's Chemotherapy Screening Program.

The data base is evaluated by the BRM Program Selection Committee, who may decide to admit the candidate BRM into the Development Network directly or to submit it first to confirmatory screening and then admit it to the Network. Based on information available in the data base or coming to the Selection Committee through feedback from the BRM Program Decision Network Committee, or from investigator-initiated research on BRM, the Selection Committee may propose that a series of new functional and/or structural analogs of a particular type of BRM be synthesized or extracted under the aegis of the BRM Program. These agents would be tested and then evaluated for admission into the Development Network.

Once a BRM is admitted, its further progression is directed by the Decision Network Committee. Depending on its state of development at the time of admission, a BRM may require isolation or synthesis on a pilot scale before it is ready for further progression through the Network. Step 3 is likely to include closely targeted developmental research leading to improvement in procedures for the isolation, purification, or synthesis of the agent. If adequate information on isolation, purification or synthesis, and BRM activity is available in the original data base, the agent is expected to be sent directly (Pathway C_1) to Procurement and Scale-Up Resource (step 4), where it is to be procured from sources outside the BRM Program, or to be "scaled up" under the aegis of the Program, if adequate amounts of it cannot be readily obtained from independent outside sources.

If the agent is available from outside sources and adequate information on isolation, purification, or synthesis is available in the data base, but information on BRM activity is insufficient for authorities to make the decision to procure it in bulk amounts, or to scale it up further within the BRM Program, only a limited amount of the agent would be procured in step 4, and the agent would be subjected to further biological evaluation (step 5) before the decision is made regarding procurement or production in large amounts. If the agent is prepared on a pilot scale in step 3 and adequate information on BRM activity is available in the data base, it may be sent directly to step 4 to be scaled up in bulk amounts within the Program, based on the information acquired in step 3. If adequate information on the BRM activity of the agent made in step 3 is not available, the product is subjected to further biological

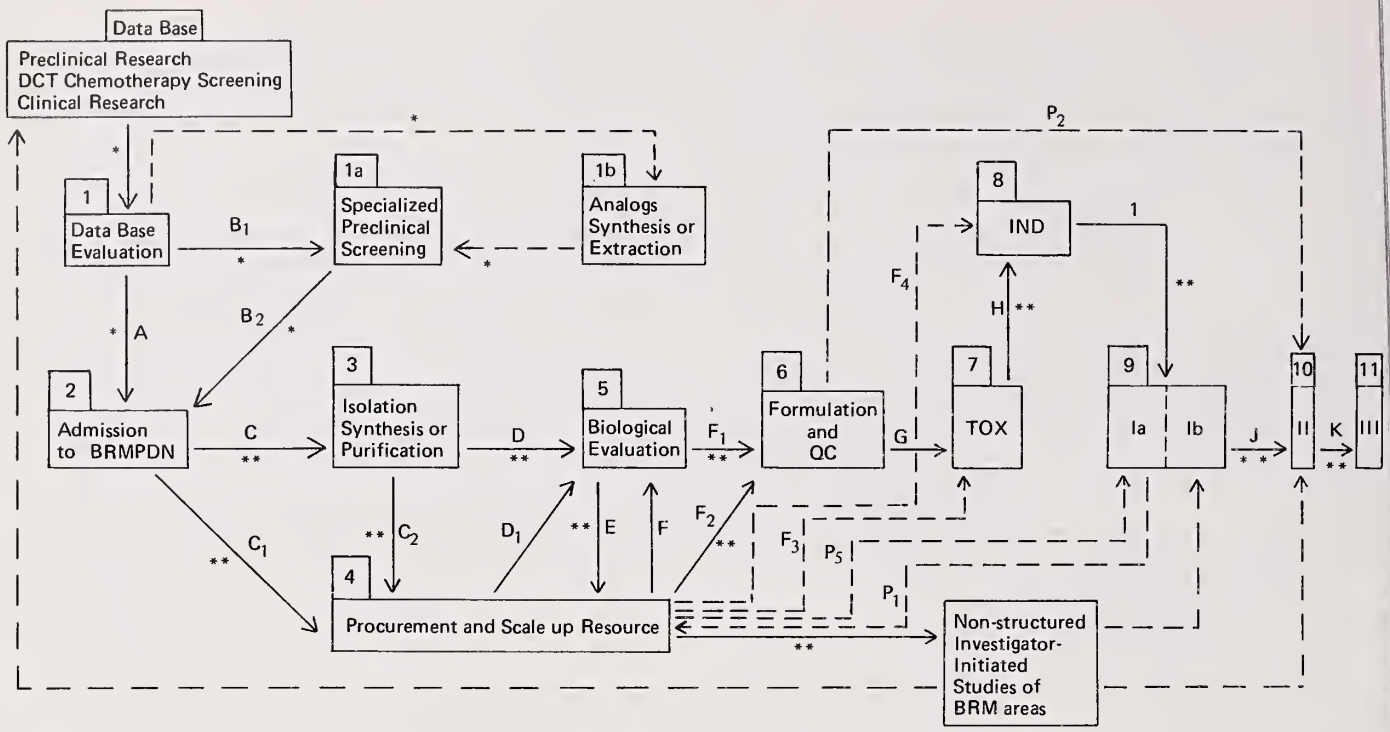

FIGURE 1-1.—Schematic outline of the BRM Program Development Network (BRMPDN). DCT = Division of Cancer Treatment; QC = quality control; TOX = toxicology; * = actions by the BRM Program Subcommittee; ** = actions by the BRMPDN Subcommittee.

evaluation (step 5). We expect that after the thoroughly studied compounds now available are introduced into the Program during its early years of existence, most agents would flow from step 2 to step 5 by way of step 3 or through limited procurement in step 4 before the decision to proceed with bulk procurement on scale up in step 4 can be reached.

Because step 5 is a complex step of biological evaluation of BRM activity through multiple tracks it will be described separately. Step 5 is expected to be an obligatory requirement for the great majority of BRM. Bypass of step 5 could be authorized in exceptional cases only for an agent which would fulfill two basic requirements: 1) It is available in bulk amounts outside the BRM Program and can be procured in purified form from the source; and 2) complete information is available on its main BRM activity, as well as on the BRM activities evaluated in the various tracks of step 5, and this information is available not only for pilot batches but also for the bulk compound which is procured for further progression in the Network. In such exceptional cases, it is conceivable that an agent could proceed from step 2 directly to bulk procurement in step 4 and from there to formulation and quality control in step 6.

Normally an agent, after having been evaluated in step 5, would return to step 4, but this time for procurement in bulk amounts. After the agent has become available in bulk amounts, it would return to step 5 for selected verification of main BRM activity and would then move to step 6. It is conceivable that in the most exceptional cases an agent might be available from the source with acceptable formulation and toxicology data or even with an IND: In such an instance, all preceding requirements having been fulfilled, an agent could bypass step 6 and move directly to step 7, 8, or 9. These bypasses are indicated so enough flexibility is included in the Network for accommodation of theoretically feasible exceptions, but it is unlikely that in practice step 6, 7, or 8 could be bypassed. In step 6, the agent would be formulated into preparations meeting the requirements of clinical trials. A targeted research component closely related to the formulation process would need to be included in this step for most of the agents. Quality control tests performed in this step would include assessment of purity, stability, and main biological activity.

After completion of step 6, the agent is expected to move to preclinical toxicology (step 7). Despite the fact that some BRM may be species-specific in their main biological activity, they should all go through toxicology for identification of potential side effects; determination of blood level kinetics should be strongly encouraged at this stage so that correlations of BRM activity, kinetics, and toxicities may be drawn and provided to the clinicians as an additional basis for reaching more rapidly optimal treatment regimens.

After step 7 is finished, the agent would move through IND procedures (step 8) and would then move to phase I trials (step 9). The phase I trials should be done in at least 2 institutions and should be performed according to classical phase I trial procedures (phase Ia) which would, however, be modified to include selected monitoring of the biological

responses the BRM is expected to modify (phase Ib). Phases Ia and Ib are not two independent steps but are two aspects of the same step that have been individually identified for emphasis on the modification of classical phase I trial procedures required for BRM. This requirement is due to the fact that BRM are expected to act by virtue of a biological activity that may be totally or partially divorced from their toxicities. Consequently, determination of regimens for phase II trials must take into consideration the relationships of dose to BRM activity. This requirement is particularly critical because, by their nature, BRM are likely to act primarily in conjunction with other forms of therapy, and thus the evaluation of their therapeutic activity must be made at regimens known to exert the expected modification of biological response. The assays to be used in this monitoring activity are suggested in a separate section below. With the major thrust of the clinical program being phase I–II clinical trials of BRM, it is suggested that task orders be generated for identification of clinical research groups capable of performing phase I–II clinical trials of BRM which, besides having adequate numbers of patients, would also have qualified clinical investigators, clinical research laboratories, and statistical personnel to perform the necessary trials with appropriate monitoring.

After phase I has been completed, should sufficient amounts of product be available, the agent would move directly to phase II trials (step 10). Should additional amounts of product be required, the agent would move to step 10 by way of a secondary production loop. Phase II trials would be performed with types of patients selected according to specific criteria that would be determined for each kind of BRM according to the type of disease and stage, status and level of the response to be modified that is usually found in that disease type and stage, and the therapy that would be used in combination with BRM under study. These trials should be rigorously designed and should include direct comparisons of combined therapy plus or minus BRM. In some instances, large numbers of patients would have to be included in the trials if we are to avoid reaching premature or erroneous conclusions. Although sometimes it is conceivable that the BRM might exert antitumor activity by themselves, at the present status of knowledge, it should be considered unlikely that BRM alone would be so effective as to justify being tested alone in phase II trials. This possibility cannot be excluded a priori, however.

After phase II trials have been completed, phase III trials (step 11) would be initiated following customary criteria. Both in trials in phases II and III, however, the specific response expected to be modified should be monitored in individual patients, at least until the time optimal regimens are unequivocally established.

Preclinical Screening of Biological Response Modifiers (Step 1a)

This step includes the confirmatory screening of BRM identified in the data base which require such tests and the comparative screening of BRM of the same general type that might be required prior to the selection of the best agent or set of agents to be introduced into the Network. This step should include tests apt to identify effects on T-cell, B-cell, and macrophage function and antitumor effects on a Signal Tumor Panel.

Procurement and Scale Up (Step 4) as a Program Resource

Step 4 in the Network is not only a necessary one in the development of a BRM toward clinical trial, but it should also represent a resource providing a BRM to that portion of the Program that is to be investigator initiated. The availability of this resource should be advertised and would represent a meaningful bridge between the Division of Cancer Treatment and the scientific community. The resource should be open to investigators essentially at two stages of development of a BRM, i.e., 1) during the early stages when a need might exist for additional basic information that could not be obtained other than through investigator-initiated research and that could be essential for the optimal development of the agent; and 2) during the trials in phases I and II when additional preclinical or clinical investigator-initiated research may yield substantial means of improving the utilization of the agent based on the additional information obtained.

All qualified investigators would be candidates to utilize this resource, including investigators who are not supported by the Division, NCI, or National Institutes of Health through grants or contracts, but who, by reason of unusual and unique expertise, would provide input into the Program, both in its structured and investigator-initiated segments.

The allocation of product from this resource should be based on a formal application; no budget request would be made but assurances would be given that funds to do the work proposed would be available. These applications would be evaluated for competitiveness of scientific quality. Specific deadlines for submission of applications would be set up to allow for critical comparisons among requests; the advertisement of these deadlines would provide an additional means of capturing the cooperation of top investigators through their participation in the investigator-initiated portion of the Program.

The BRM Subcommittee of the Division's Board wished to be on record with a strong recommendation that this resource be established and used as indicated above.

Biological Evaluation (Step 5)

This component of the Network would include 6 primary tracks (fig. 1-2), i.e., a common track in which all BRM should be evaluated and 5 specialized tracks, one each for: 1) IF, IF inducers, and other cytokine products; 2) thymosins and thymic factors; 3) immunomodulating agents; 4) tumor-specific treatment; and 5) cancer preventive agents. Some IF inducers are augmenting agents and vice versa.

The concept underlying the design of this step is that BRM with an expected BRM activity may also have unexpected specialized modifier activities. Thus each agent should undergo primary evaluation in the track reflecting its main putative BRM action and, at the same time, in the common track. This common track should include selected

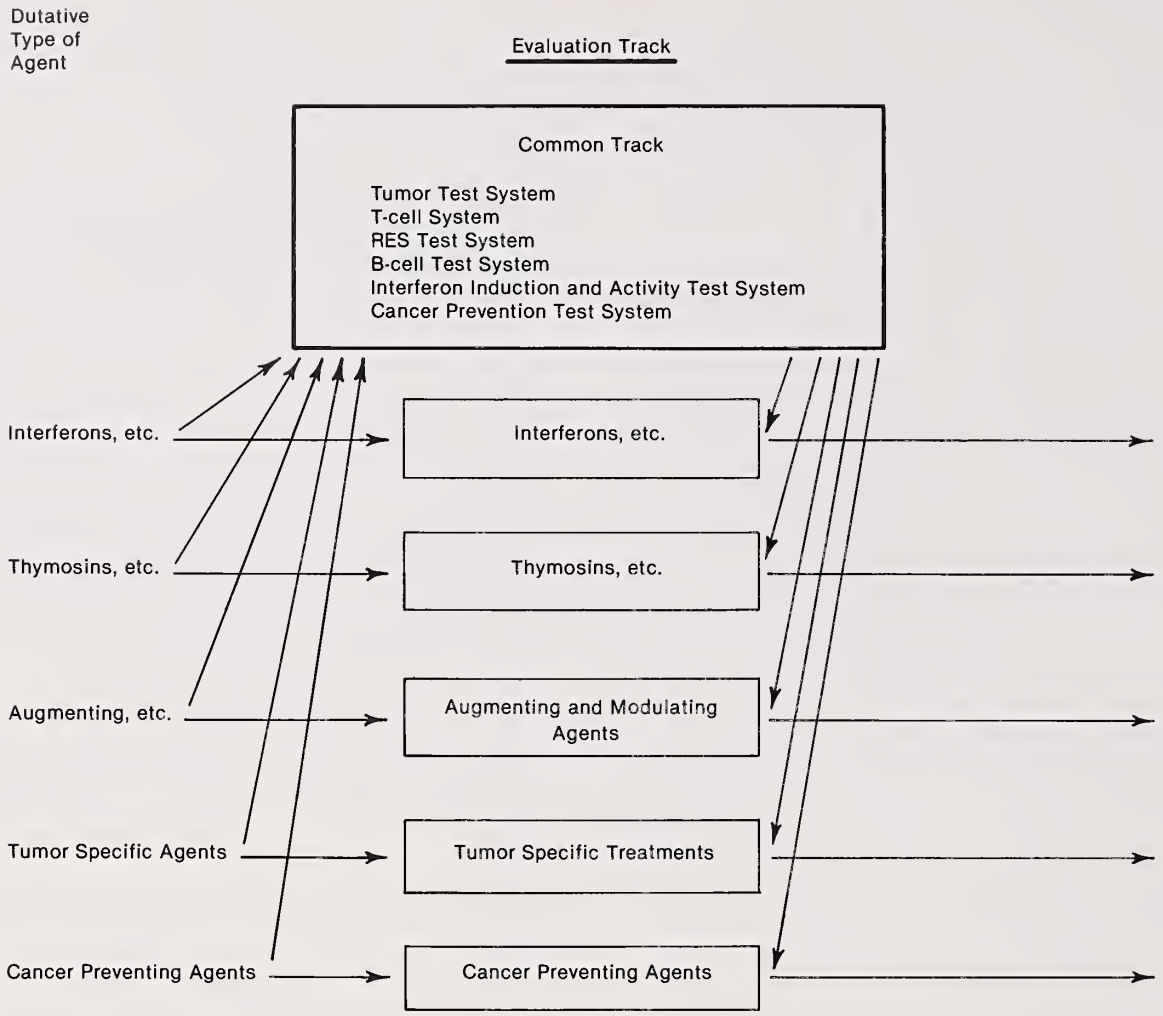

FIGURE 1-2.—Biological evaluation (step 5).

signal tests apt to identify other unexpected BRM activities. Those agents which would show unexpected activities in the common track would then undergo secondary evaluation also in the specialized track(s) pertaining to the unexpected activity found. Identification of the multiple primary BRM activities of an agent within this Program is important because the agent would affect specific aspects of its clinical evaluation.

The common track.—This track should include the following types of signal tests: 1) a small tumor screening to identify antitumor effects mediated through host responses; 2) a T-cell test system to detect modifications of T-cell functions; 3) a B-cell test system to denote modification of B-cell functions; 4) an RES test system to identify modifications of macrophage function; 5) a test system for IF for its induction and action, and 6) a cancer prevention test system to pinpoint an agent with such activity. We stress that all the tests suggested in this Report are not meant to represent rigid directives but constitute suggestions about the type of functions that need to be evaluated: The investigators involved in the actual work should be given the flexibility to propose the test they would consider to respond optimally to the evaluation needs.

Tumor screening.—This system should be developed according to the following specifications: 1) comparison of potential signal tumors for their suitability to identify BRM (ultimately no more than 3 systems should be selected); 2) dose–response experiments for experimental designs determined for optimal visualization of therapeutic and/or prophylactic effects of BRM; 3) selection of the order of priority of the tumor systems and tests compared, with reference to identification of end points for evaluation of BRM activities in the tumor systems and the effects of the various classes of BRM agents; 4) testing of several of the prototype BRM available not only for test system development but also establishment of standards for future reference.

T-cell system.—A standard allogeneic tumor cell system may be used. Test agents should be given to the responding mice at different times after immunization, and the assay should be run at different times. Two immunizing doses should be used, one giving an optimal response in control mice and the other a suboptimal response (under these latter conditions, augmenting effects are more likely to be visualized).

B-cell system.—A standard Jerne's direct PFC assay may

be performed. Modifications of the choice of test system and/or conditions should be a prerogative of participating investigators.

Macrophage activation test.—Several methods for in vivo RES activation have been developed. Of the many tests available, most of them, including mononuclear enzyme induction, correlate well with the quantitative clearance of particles from the circulation. The specific particle used is not critical as long as standardization is sufficient for reproducibility.

Interferon and other cytokines.—Because IF and its inducers may also stimulate the RES and NK cells, tests for IF activity evaluation should be compared with those for macrophage and NK cell activation. Based on the known species specificity of IF and in view of doubts about the generality of its induction by agents, only human cells should be used to test for IF activity itself. Two main assays should be compared, i.e., a cellular cytotoxicity assay and a tumor response-prophylaxis assay. A cellular cytotoxicity in vitro assay should be developed with suitable human tumor cell lines as optimal target cells for IF. The tumor response-prophylaxis assay should be developed for comparison of the suitability of 3 basic tests: 1) effects on contract inhibition in a suitable human cell culture system, 2) effects on the capacity of human tumor cells to form colonies in semisolid medium, and 3) growth inhibition of human tumor cells in culture. The details of the experiments should be determined by the investigators developing these tests.

Cancer prevention test.—In vitro testing is not recommended now except for retinoids because the response to chemical carcinogens of all currently available test systems is not sufficiently well understood at this time. In vivo test systems should involve the chemical induction of carcinomas in a mouse model. Because of the ubiquitous nature of the nitrosamines and/or nitrosamides and the faet that they induce a wide variety of epithelial tumors, the effects of putative chemopreventive agents may be evaluated in mice previously exposed to such carcinogens. A second system which would avoid the use of chemical carcinogens would be an assessment of the effects of BRM on the spontaneous induction of a neoplasm in a high-incidence mouse model.

The specialized tracks should be used *concurrently* with the common track for all putative BRM of these general types and *subsequently* to the common track for those other agents that have potentiality for this type of BRM action during evaluation of the Common Track Signal Test.

The IF, IF inducers, and lymphokine track should include the following systems in which tumor cells would be used: 1) antiviral assay, 2) assay for each known lymphokine activity, and 3) assays for effector cell activation. In addition, because this type of BRM can also be reasonably expected to modify certain immune functions, *all the agents* with this putative action should be evaluated in the augmenting agents and thymosin tracks. The antitumor cell activity would be evaluated within the common track.

The thymosins and thymic factors track should include assays apt to measure the functions indicated: 1) Changes in enzymatic and surface antigen markers of maturational status should be measured. As T-cells undergo maturation, changes in antigens expressed on their surface as well as changes in certain intracellular enzymatic levels are measurable. These alterations are best characterized in murine systems and represent excellent methods of following changes induced in maturing T-cells by exposure to thymic hormones. 2) Functional changes in T-cells following exposure to thymic hormones need to be measured. As T-cells undergo maturation, functional changes in the ability of the cells to react to stimuli are apparent and have been measured in various ways; they have been induced in vivo and in vitro. It is extremely important that demonstration of functional changes be a part of the analysis of purified thymic hormones and factors. Many functional assays have been reported over the last 10 years for thymosin and the other thymic factors. They include assays to measure GVH, MLR, MIF, CML mitogen responses, PFC responses, suppressor cells, tumor growth, and others. Because none of these functional capabilities are totally eliminated by adult thymectomy, the relative importance of these assays cannot be fully evaluated now. However, the thymic factors being evaluated should be tested in various assays in vivo and in vitro with animal models having compromised immunological systems. The experimental model will vary, depending on the specifics of the assays used, but will include modification of immunological responses.

In the augmenting, immunomodulating, and restorative agents track, the tests to be used are those which would identify agents meeting the following criteria: 1) The agent will restore or augment some biological response parameter in animals. This parameter will be one which is of some consequence in host control of tumor growth. 2) The agent should have demonstrated antitumor effect in animals in a clinically relevant model. The antitumor effect should be related to modification of the biological response modified [as in (1) above]. In addition, when possible, a test should be available that can measure this biological response parameter in man.

For example, in this track, functions to be measured would be those of T-, B-, and NK cells; macrophages; suppressor adherent cells; and other general tests for delayed sensitivity or resistance to infection.

The tumor-specific preparations track should include assays capable of measuring 1) immunizing activity of tumor antigens, modified or not, 2) cytotoxic effects of antitumor-associated antigen antibodies, and 3) specific antitumor effects of activated lymphoid cells. This track is not represented in the common track because of the selected and specialized nature of the agents to be evaluated. At a later date, a Common Track Signal Test that would help evaluate agent-related modifications of tumor antigens may be developed.

In the retinoid track, investigators would select the tests to be used based on the information acquired on the development of these tests. The following in vitro screens or their equivalents are recommended: The mouse prostate system measures the ability of retinoids to reverse carcinogen-induced hyperplasia and metaplasia in mouse prostate organ culture. The inhibition of SGF measures the inhibitory effects of retinoids on the response of cultured

fibroblasts to the phenotypic transforming activity of SGF. The hamster trachea system has shown an excellent correlation between a positive response in inhibiting keratinization of trachea organ cultures and the ability to inhibit carcinogenesis in vivo. This assay measures vitamin A-like activity.

As the activity of retinoids in inhibiting carcinogenesis does not appear to be related directly to their toxic properties, compounds showing low toxicity and high activity as inhibitors of transformation should be identified in the in vitro assays and tested further in experimental animal systems.

In vivo localization of drug in all major organs should be examined with emphasis placed on those which are a frequent site of cancer in man.

The biological action of localized retinoids should be examined in a rodent system in which tumors of that organ can be readily induced. Because of their great diversity of organ specificity and their apparently ubiquitous presence in the environment, the nitrosamines appear to be the most useful agents for inducing clinically relevant carcinomas. The experimental protocol should include a group of animals in which the tumor incidence approximates 10–20%, so that the activity of these agents may be evaluated at 1) a more clinically relevant tumor incidence range, and 2) at a carcinogen dosage level that does not cause major nonspecific organ toxicity.

Phase Ib (Step 9)

With BRM, the phase I trials should include an essential component providing adequate monitoring of the biological responses that the BRM under study is expected to modify. The monitoring systems suggested for implementation are briefly outlined below according to BRM type.

The monitoring of IF action might require in vivo measurements of modification of immune responses based on the assumption that these IF effects are an adequate signal of antitumor activity. The effects of IF on the NK and ADCC activity of PBL may be among the most reliable methods for detection of activation of host defense mechanisms by these agents. In addition, the recently described inhibition of complement fixation reactions may also be followed serially. Measurements of IF levels in serum and in other substances including spinal fluid, urine, and effusion should also be taken.

The monitoring of activities of thymosins and thymic factors would be performed with assays based on tests on PBL.

Measurement of T-cell numbers and subpopulations can be accomplished with rosette assays. The degree and direction of modulation of functional responses of patients' PBL by thymosin or other factors primarily depend on the initial immunological status of the patient. Therefore, patients with depressed responses would be most likely affected by an augmentation of their responses, whereas patients with normal or hyperresponsivity would most likely show no effect or diminished (or suppressed) responses upon exposure to thymosin polypeptides or other immune regulatory factors. The assays utilizing the lymphocyte functions that are the most extensively characterized as being effective in delineating the immunoregulatory properties of thymosin polypeptides and other immunomodulating factors are the MLR, the mitogen response assays, and antibody production in responses to PWM.

Suggestions for Monitoring the Effects of Alleged Immunotherapeutic Agents in Patients With Cancer

Monitoring of immunocompetence and modification of host immunological reactivity is central to the conduct of the trials of the BRM thought to be immunomodulators. Below are listed a broad spectrum of currently available host defense assays which are reasonably reproducible and some of which are known to be modified by BRM. We emphasize that this area is still early in its development. Additional assays are under study by various investigators; assays are indicated for tumor-related and general host reactivity. Although no one test or group of tests has been entirely satisfactory in monitoring the response of the patient to his tumor, several are relevant enough to be applied serially as they undergo continued development. From the list below, investigators may select a small number of tests most relevant to the BRM under study and follow them serially. For example, for thymosin, T-cell numbers and MLC blastogenesis may be sufficient, but for augmenting agents, macrophage function tests should be more relevant. Investigator-initiated approaches should be encouraged toward the development of more suitable assays and the improvement of those available. Finally, it is both impossible and undesirable for any one group to conduct a full spectrum of assays; a focused, relevant, small group is what is desired.

Characterization of Cell Types by Their Antigens and Markers

These tests permit evaluation of changes in proportions of immunologically active cell populations: E-rosettes for T-cells, surface Ig-bearing (B) cells by immunofluorescence, Fc receptor-bearing cells (macrophages and K-cells), and complement receptor-bearing cells by EAC rosettes.

Reactivity to Nontumor Antigens

Cell-mediated immunity

Delayed skin reactivity: Primary sensitization of DNCB or keyhole limpet hemocyanin.—Recall sensitization to microbial antigens, such as varidase, *Candida,* and mumps, can be helpful in assaying general immunocompetence, though it is a less sensitive measurement of changes effected by modulators.

In vitro blastogenesis.—In vitro blastogenesis to mitogens, such as PHA, Con A, and PWM, may also be helpful to us in assessing immunocompetence and in monitoring effects of therapy.

Lymphocyte-monocyte interaction.—To determine whether peripheral blood monocytes are activated or depressed in their function of supporting or suppressing lymphocyte blastogenesis and to determine whether deficient lymphocyte function can be corrected by removal or addition of monocytes, scientists can conduct blastogenesis

experiments, as described above, in response to PHA, Con-A, and PWM with 3 types of cultures: patient leukocytes plus normal monocytes; normal leukocytes plus patient monocytes; patient lymphocytes depleted of monocytes by passage of cells through glass bead columns, nylon fiber columns, or by incubation with carbonyl iron followed by Ficoll-Hypaque density solution centrifugation. Calculated responses then indicate whether or not suppressor cell activity is present.

Assay for MLC-CML.

Assays for ADCC to SRBC (K-cell activity) or human RBC coated with antibody (monocyte activity).

Serum muramidase activity.—This is an important method because in animal models, infiltration of tumors with large numbers of macrophages is associated with an elevation of the serum lysozyme level and with subsequent tumor regression. Also, BCG and *C. parvum* increase the serum levels of lysozyme in animals, which therefore reflects macrophage activation.

Humoral immunity

The primary antibody response to a bacterial toxoid such as tetanus or to Vi antigen or keyhole limpet hemocyamin is measured by microtitration. Dissection of the IgM and IgG components of the response, through 2-ME sensitivity or a more detailed analysis on columns or gradients, provides further useful information. Measurement of the secondary (anamnestic) response to protein antigens, such as toxoids, is less sensitive than the primary response because many memory cells are already established but may be useful. Note that these serological measurements reflect a composite of effects on T- and B-cells and macrophages.

Reactivity to Tumor Cells

Cell-mediated cytotoxicity

Although a controversial subject, cell-mediated cytotoxicity against autologous tumor cells has been used successfully by several investigative groups, and strict attention to techniques of tissue culture, preparation of effector cells, effector-to-target cell ratio, and labeling with radioisotopes have made the test more reproducible and unbiased recently. Whether this test reflects the status of the patient toward his tumor is debatable, particularly if the effector cells are those in the peripheral blood, but it is conceivable that lymph node cells or those obtained from the tumor bed might be a more accurate gauge.

Scientists have found that NK reactivity, activated monocyte-macrophage cytotoxicity to tumor cells, macrophage-mediated cytotoxicity, or cytostasis against autologous tumor cells, ADCC reactivity related to macrophage-mediated cytotoxicity, and LAI, reflecting a macrophage-mediated reaction in man, may be helpful in judging the effects of immune response modifiers.

Humoral immunity

Either LAI or ADCC tests for CMI could be used as an indirect test for humoral immunity, with preincubation of a patient's serum with either target cells (ADCC) or the effector macrophages (LAI).

Although cytophilic antibodies have been demonstrated in several diseases, it is possible that a less cumbersome adaptation of methods for their demonstration, such as through the LAI test, will facilitate their routine measurement by many investigators.

Measurement of cytotoxic and cytostatic antibodies in patients has proved extremely difficult. Complement-fixing and fluorescent-tagged antibodies might be demonstrated, but few data show that either this is possible or that, if demonstrable, the antibodies are directed to TAA. Some of the ostensible tumor-specific, cross-reactive fluorescent antibodies shown in the past have proved to be tissue culture components or contaminants.

Operating Committees

Biological Response Modifier Program Selection Committee

The functions of this Committee should be as follows: 1) accept agents for introduction into the BRM Program Network in limited numbers, after critical evaluation of their properties and Program priorities; 2) decide whether candidate agents are to be introduced into the Network directly or upon confirmation of preclinical BRM activity under the aegis of the Program; and 3) decide whether analogs of candidate agents should be proposed for synthesis under the aegis of the Program. Generally, the criteria for acceptance should be demonstrated BRM activity, adequate characterization by source and batch (chemical characterization is not required), the agent's activity must be assayable and quantifiable, and the data base activity results must be consistently reproducible.

Biological Response Modifier Program Decision Network Committee

The functions of this Committee should be as follows: 1) evaluate the progression of BRM along the Network, 2) make decisions related to this progression both in development toward clinical trials and of direct versus bypass pathways to be followed, 3) evaluate the desirability of moving BRM to step 6 based on a critical analysis of the information available at that time and the comparison of the candidate BRM with other BRM with known activity on the same biological response, 4) evaluate with particular care the reproducibility of the data required at each step, 5) allocate procured or scaled up BRM (in step 4) to further work along the Network versus to work in the investigator-initiated areas of the Program and evaluate applications for a BRM supply submitted to the Division of Cancer Treatment, 6) define signal tumors to be used in phase II and select BRM assays to be used in phase Ib and phase II, 7) allocate BRM quantities to clinical groups and recognize needs for additional procurement, 8) formulate recommendations for Program interactions primarily in relation to the chemotherapy and radiotherapy programs during phase II, and 9) determine periodically which Workshops and Study Section Briefings may be needed to accord the BRM Program community awareness and fair evaluation.

PROGRAM ADMINISTRATION

Because of the multidisciplinary nature of the BRM Program and the complexity of its operations, particularly

as they are related to evaluations, assays, and decisions among alternative choices in the development of the agents, it is important that the Program be led by a single Director who would oversee all its aspects.

The number of agents to be processed is almost inversely proportional to the complexities of their development. Thus it does not seem indispensable that a separate subdivision of the Division of Cancer Treatment be set up, in view of the fact that scientists at several of the existing laboratories could be involved in implementing parts of the Program with appropriate additions and modifications and under appropriate leadership. Therefore, the Director of the BRM Program should be administratively responsible directly to the Director of this Division and should be assisted by two special assistants attached to the offices of the Directors of the Cancer Therapy Evaluation Program and Developmental Therapeutics Program, respectively, but administratively responsible directly to the Director of the BRM Program. Alternatively, the Director of the BRM Program should be hired at the level of Associate Director in charge of a separate Cancer Treatment Program with a small intramural facility allocated directly to the Program, notwithstanding the relationships to the two Programs mentioned above. This alternative would provide the BRM Program with the necessary administrative identity.

The Director and his or her two special assistants should work closely with the Directors of the Cancer Therapy Evaluation and Developmental Therapeutics Programs. To provide necessary flexibility, identity, and leverage to the Program, regardless of which of the above two alternatives is chosen, it should have its own budget, probably through the Office of the Director of the Division if other devices are administratively inappropriate. The Director of the BRM Program would allocate budgets to Program components, directly or through the Cancer Therapy Evaluation or Developmental Therapeutics Programs as needed.

The Subcommittee on BRM of the Cancer Treatment Board wishes to be on record with the request that a new Study Section of the Division of Research Grants or a grant evaluation committee of NCI be set up to evaluate BRM Program grants. The Program is heterogeneous in its component parts, and no presently available Study Section seems appropriately staffed to evaluate grants within the total scope of the Program. The Subcommittee also proposes that workshops and presentations be scheduled to introduce the new Program to the members of those study sections which would be more closely related to the BRM Program. These efforts are deemed necessary because, in addition to its Network components which can be best supported with contracts, the Program must rely heavily on investigator-initiated research.

In further efforts to introduce the BRM Program to the scientific community and to elicit the interest of the best of its members, representatives of the Program should organize (under the aegis of the Program) and schedule a series of specialized workshops during the first 2 or 3 years of its existence.

PROGRAM FOR INITIAL IMPLEMENTATION: GENERAL RECOMMENDATIONS

The program proposed for the first year included:

A) Establishment of the BRM Program and the Program administration,
B) Implementation of the specific programmatic recommendations,
C) Initiation or continuation of several lines of preclinical developmental research under the aegis of the Program.

Parts A and B are expected to be funded predominantly from the intramural budget and contracts and through responses to RFA, and Part C from an appropriate combination of contracts plus R0-1 and Program Project grants responding to RFA, as well as through stimulation of the scientific community that will lead to increased investigator-initiated research in the BRM areas. Part A was described earlier in this Section. Parts B and C are outlined below, as they represent the continuous implementation of the Program. A few specific recommendations for accomplishment of the proposals are indicated.

Interferons

The IF project should be divided into 3 major parts: clinical trials and related studies, preclinical research and development, and production. Although production will be the first project to be implemented, the production needs necessarily depend on the first two sections.

Clinical Trials and Related Studies

As outlined above, each BRM should be developed clinically through modified phase I and then phase II trials. Clinical IF trials conducted to date independent of the BRM Program have been performed with doses and regimens selected empirically, based on the availability of the agent. Optimal doses and regimens for IF must be identified through appropriately designed phase I trials. As with all other BRM, phase I trials with IF should include not only the tests for toxicity but also those that monitor modification of biological responses, so that optimal doses may be selected for both toxicity and biological response modification. At this time, the only type of measurement of biological response modification that appears practical for IF is a measurement of the effects on immune responses and especially on NK activity. Possible age-related differences in effects must also be considered. Pharmacological studies should evaluate the blood kinetics of IF by appropriate measurements in vitro with bioassays for antiviral and cytostatic effects.

Although phase II trials customarily follow and depend on the results obtained in phase I trials, phase II trials for IF are already being performed independently of this Program. It is recommended that phase I studies be done as soon as possible because optimal doses are urgently needed for rigorously designed phase II trials.

Formulation and quality control development research is directly related to the clinical development of IF and should be pursued so that the various IF preparations can be standardized at different stages of purification.

It is essential that purified preparations of IF be tested clinically as soon as possible for determination of the therapeutic advantages they may provide. This need is especially important in view of the fact that the clinical antitumor results obtained to date have been obtained with relatively impure material. Considerable developmental groundwork as well as scaling up procedures that are under development in pilot research laboratory form are required.

Preclinical Research and Development

We need substantial information on the basic mechanisms of antitumor action of IF and on ways to improve the therapeutic usage of these agents.

The mechanisms of inducer-cell interactions and of induction of IF need clarification. The mechanisms involved in the regulation of IF production, as can now be studied at the transcriptional and translational levels, should be explored. The phenomenon of superinduction which can help in these studies itself needs to be understood better, and, if possible, exploited in non-fibroblast-type cells because its general exploitation could lead to higher IF yields from lymphocytes whose IF is presently used experimentally in the clinical setting.

Studies with IF mRNA, which can be tested with great sensitivity in biological assays involving their translation in heterologous cells, should be pursued. They lead to the synthesis of cDNA for IF that allows recombinant DNA technology to be applied toward developing methods for the industrial scale production of IF in "bacterial factories."

The complete purification of several IF has been achieved, although not on a preparative scale. Achievement of the latter should be an urgent goal because the field would benefit in several ways. The preparation of highly specific antibody would itself contribute to efficient affinity chromatography purification methods. Pure mouse IF preparations and specific antibody would allow studies of the role of the IF system in animal models and clarify the part it plays in protection and recovery from diseases, either infectious or neoplastic. Pure human IF preparations will also allow the complete characterization of the IF molecules for their physicochemical and biological properties in vitro and in vivo. The deglycosylated IF polypeptide of human and mouse species, which is already known to be completely biologically active, can be studied for its amino acid sequence, or it can be degraded enzymatically or chemically, when one attempts to obtain an "active core." Knowledge of a partial or complete active sequence can also lead to an application of recombinant DNA techniques. Alternatively, chemical synthesis of the IF polypeptide itself can be attempted that could also result in low unit cost in industrial production. A task of immediate urgency with regard to the above is the production of sufficient amounts of deglycosylated mouse and human IF, or active fragments, so that their pharmacological properties can be evaluated in vivo.

Various human IF need to be studied with regard to their possible tissue specificities, knowledge of which would have immediate clinical applicability. The presence of a specific receptor for IF on target cells should be verified by the isolation of such molecules and expanded through a study of its binding characteristics.

New gene products have already been identified in IF-treated cells expressing an antiviral state. One cannot assume at this time that the antiviral and antitumor effects of IF are mediated by entirely different mechanisms. Among IF activities directly observable in cells in vitro, the growth inhibitory effect may be directly related to the antitumor effect observable in vivo.

Of particular relevance and interest to the BRM Program are the many immunomodulatory effects of IF of the B- and T-cell-mediated immune responses. In addition, enhancing effects of NK cell and macrophage functions have been reported in man, in vitro, and in animals as a result of IF treatment and within a context that suggests that such effects may be partly responsible for IF-induced but host-mediated, antitumor activity. The lines of investigation implied above and others that should be pursued as seen by the scientific community at large may often best be conducted with human IF, but many also have to use mouse IF in the mouse, so that concepts that may be verified later in humans can be developed.

Production

The large-scale production of all three types of IF has now become feasible and should be vigorously pursued if we are to obtain the quantities of IF required to do the preclinical and clinical studies recommended.

Based on the proposals mentioned above, it is likely that at this time about $270{,}000\times10^6$ IU *of each type of human IF* are needed. These would be used roughly as follows: phase I: $15{,}000\times10^6$; phase II: $150{,}000\times10^6$; formulations, etc.: $5{,}000\times10^6$; purification and phase I: $50{,}000\times10^6$; and other research: $50{,}000\times10^6$. Because allocation of the required amount of money to the IF production portion of the BRM Program is impossible, plans have been scaled down, so that the IF production would be more appropriately related to the other important parts of the BRM Program during the initial phases of its development when all its central features must get a solid start. Specifically, we recommend that IF production be initially geared at the following scaled-down plans: 1) 3 phase I trials; 9 phase II trials; formulation, purification, and research; and phase I trials with purified IF would be planned with HuLeIF; 2) 3 phase I trials; 1 phase II trial, and purification research would be planned with HuLeIF; 3) only research would be initially done with human fibroblast IF; 4) Type II human IF would be produced for initial research; and 5) mouse IF would be used for development of other studies leading to the clarification of important research questions. These plans could be modified further depending on the information acquired and the availability of products obtained through genetic engineering.

Thymosins

The projects proposed for the development of the thymosins fall into 3 major categories: clinical trials and related studies, basic development and related studies, and production.

Clinical Trials and Related Studies

Phase I trials of both thymosin fraction 5 and thymosin α_1 should be conducted during the first year of the Program, and phase II trials should be initiated only when a regimen can be designed using doses proved in the course of the phase I trials to have restorative capabilities with respect to T-cell numbers and function.

Inasmuch as thymosin acts in an immunorestorative manner rather than having a direct antitumor effect, a primary prerequisite for initiation of clinical trials should be documented immunosuppression, due either to the direct presence of the tumor or the therapeutic regimen, i.e., chemotherapy, radiotherapy, and/or surgery. Responsiveness to thymosin would require that there be some bone marrow reserve and the presence of thymosin-responsive cells in the blood (in vitro assay). An appropriate trial setting would be measurement of the capacity of thymosin to reconstitute or maintain T-cell immunity in immunoincompetent cancer patients.

Phase I studies should also include pharmacological evaluation of the kinetics of thymosin, i.e., serum and tissue half-life. Either RIA or ELISA for the polypeptide factors will be used as they are developed. When those assays are not available, scientists will quantitate serum levels by bioassay using the murine azathioprine rosette assay of Bach and/or the murine Thy-1 assay of Twomey. The RIA are already available for some of the thymic peptides such as thymosin α_1 and thymopoietin.

Concurrent randomized controls not receiving thymosin fraction 5 and thymosin α_1 are mandatory because of the variations in human immune reactivity. Studies should be done first with patients not on other therapy but subsequently could be done in patients on cytoreductive therapy.

Basic Developmental Research

Further studies with components of thymosin fraction 5 as well as comparative evaluation and development of other known thymic factors which may offer additional selective therapeutic advantages are recommended. The factors that have been reported to give significant biological activities, in addition to the thymosins, include THF, FTS, thymic factor, thymopoietin, and the prealbumin thymosin-like factor. An additional number of factors have been reported (*see* Section IV) that may prove to be clinically useful in the future.

Study of the role of thymosin in stem cell differentiation, maturation, and function; induction of markers.—Initial studies have demonstrated that several of the thymic preparations influence expression of T-cell markers and functions in some marrow stem cell populations. Now that purified and synthetic thymic peptides are becoming available, these preparations should be investigated for their potential to induce T-cell differentiation in purified stem cell *subpopulations.*

Emphasis should also be placed on the biochemical events leading to stem cell differentiation. Analysis of surface membrane and intracellular metabolic and enzymatic changes, resulting from exposure to thymic hormones and in evidence of T-cell differentiation, should be performed, e.g., induction of cyclic nucleotides, alterations in Ca^{2+} transport, changes in protein synthesis, etc.

It is important that the various thymic peptides, such as the components of thymosin fraction 5, be studied in sequence and in combination for analysis of the maximum potential for induction of stem cell differentiation along the T-cell pathway. Several peptides may act on different stem cell subsets in multiple stages to induce different T-cell pathways. Because many of these studies can be performed in vitro, these approaches should be conducted with human and animal cell models.

For the induction of functional T-cells, thymosins should be studied for their capacity to induce functional alterations in lymphocyte reactions that can be explained by lymphocyte maturation. Special emphasis should be given to restoration of defective lymphocyte function in vitro in cancer patients and in vivo and in vitro in tumor-bearing animal systems.

Role of thymic hormones in normal differentiation and maturation of functional T-cells.—In vivo, the thymus most likely functions at a minimum of 3 levels by inducing: 1) differentiation of incoming stem cells to form immature thymocytes, 2) immature T-cells to become mature T-cells in the thymus, and 3) maturation in the periphery by elaborating circulating thymic hormone(s). We need to establish the full role of the thymic hormones in stem cell development. A second important area of basic research is the question of how thymic peptides act in situ in the thymus and how and where is hormone synthesis regulated.

Priority areas of research should include: 1) identity of the (epithelial) origins of the various thymic hormones, either by fluorescence or radioautographic techniques, with the use of antibodies raised against the thymosin peptides. Studies with cultures of thymic epithelial cells of animal and human origin will be useful in these studies. Questions such as the homeostatic control of hormone synthesis need to be addressed, e.g., how do the other endocrine organs influence thymic epithelial function and synthesis of thymic hormones. Consideration should also be given to the influence of ontogeny, senescence, and clinical states on such function and synthesis. Attention should also be directed to thymic epithelial function and control in abnormal states, such as thymomas [e.g., human or murine (AKR) in which thymic epithelial malfunction may be a key component to the pathogenesis of lymphoma].

Further information is needed on the influence of thymic hormones and individual peptides on thymocyte subpopulations and the intrathymic differentiation pathways. One can use the same criteria for T-cell differentiation and function, as described for stem cell studies above, to evaluate mechanisms of thymic peptide action. Again, studies with human and animal cells are desirable.

With the thymus functioning as an endocrine organ, more basic research is needed on the levels and forms of thymic hormones (precursors, degradation products) in the circulation, as well as on the peripheral target cells of the circulating products.

Influence of the thymosins on states of immune imbalance.—Ultimately, the basic research on thymic hormones should lead to therapeutic applications in clinical disorders with immunological components. Among the

areas in which more basic research is needed are: tumor growth, autoimmune disorders, aging, and infectious diseases. Above-mentioned research on stem cells and thymocyte differentiation and maturation will also be directly applicable to the understanding and treatment of the primary and secondary immunodeficiencies. Conversely, understanding of how thymic hormones function in states of immune imbalance should provide insights into the basic mechanisms of action of thymic hormones in the maintenance of normal immune balance and in host resistance to progressive tumor growth.

Biochemical characterization and synthesis of thymic peptides.—Considerable progress has been made in the past 2 years in the chemical characterization of several of the biologically active, purified thymosin peptides and with the active biological components of THF, thymopoietin II, and the serum thymic factors of Bach (FTS) and Astaldi (SF), and other thymic-derived or dependent factors, such as the prealbumin fraction of White. Given a potential role for these thymic factors in modulating immunity, a major area for priority development would be the support of research proposals to characterize fully and chemically the biologically active polypeptides. These studies should include purification of material in sufficient quantities for amino acid sequence analysis.

Sequence analysis of the purified polypeptides is necessary because it will facilitate comparison and the identification of similar to disparate biologically active components in the various thymic preparations. Major efforts should be given to the synthesis of biologically active and well-characterized thymic polypeptides for development of the methodology necessary for large-scale production geared to clinical applications. Classical chemical synthesis and solid-phase synthesis of thymosin α_1, thymopoietin II, and FTS have already been accomplished. Similar strategies should be developed with the other thymic factors.

Development of assays to measure levels of thymosins in the blood.—The development of sensitive RIA or ELISA of the thymosin peptides in the blood are needed so clinicians can identify patient populations and monitor therapy.

Studies with other thymic factors.—Investigations, such as those outlined above for the thymosin polypeptides, should also be performed in a similar manner for THF, FTS, prealbumin thymic factor, thymopoietin II, and other thymic factors (as they are identified). It is essential that adequate material of each of these factors be made available to the researchers working in the area of basic immunobiology so that comparisons between the different factors can be made.

Production and Support Recommendations

Support is recommended for the 1) clinical trials, 2) development of staffs in clinical resource laboratories who can conduct the immunological analyses required for these trials, and 3) laboratory personnel who are capable of providing the various thymic preparations and RIA for the necessary research activities and for performing important aspects of the basic research program, such as preparation and testing of thymosin fraction 5 components (other than thymosin α_1); development of RIA for thymosin peptides and screening of blood samples in the phase I and II trials; preparation of prealbumin thymic-like compounds; preparation of THF, SF, FTS, and thymopoietin for comparative studies; comparative screening of thymic hormones in defined tumor treatment animal models; comparative screening of thymic hormones in defined animal models of immunosuppression; and development of novel approaches for large-scale production of clinically defined thymic hormones. Procurement of adequate amounts of thymic factor and hormones would be required in the pursuit of recommended lines of investigation.

Immunomodulating Agents

Augmenting agents include those natural or synthetic products which increase the immune or host defense reactivity of the subject above the normal resting level. This area includes the so-called active nonspecific immunotherapeutic agents, the microbial adjuvants, various low and high molecular weight polymers, IF inducers, and certain other compounds. Clinical trials with augmenting agents are discussed in Section II, and some of the augmenting agents are reviewed in detail in Section V.

A review of this area has resulted in the identification of a group of agents which have antitumor activity in animal models, modify biological response in a measurable fashion, and for which monitoring methodology is available for clinical trials. One agent, suggested for initial development, is indicated below. Other agents of interest should be considered for evaluation through the operation plan of the Program.

Improved understanding of the human immune response and the development of good assays to monitor monocyte and macrophage activation and activation of other defense mechanisms permit the clinical development of these agents in a rational fashion; this should overcome some of the difficulties experienced in earlier work with augmenting agents in which appropriate monitoring was either not available or not applied. The number of augmenting agents and IF inducers which have activity in animal tumor models and measurable biological response modifying activity is large. Priority selection on the basis of available efficacy data is difficult. Nevertheless, MVE-2 was selected for the BRM Development Network; its study in a phase I trial will establish the mechanism by which an augmenting agent can be evaluated for its biological activity in man according to the experimental design and the host defense mechanism evaluation techniques recommended.

A 15,500-dalton copolymer of maleic anhydride and divinyl ether, MVE-2 is related to the parent compound pyran copolymer, which has shown antitumor activity in various animal models and in addition was used clinically in phase I trials. However, MVE-2 proved highly toxic, presumably because of its heterogeneous molecular weight and content of higher molecular weight components. It is highly purified and of defined molecular weight and has been active in animal models with markedly reduced toxicity. Preclinical toxicology studies have been conducted by Adria Laboratories. In addition to MVE-2, a series of MVE compounds, MVE-1 through MVE-5 with a spectrum of molecular weights, is available for future investigation. Clinical investigation of these agents should

await the results of phase I trials with MVE-2, which is the lowest molecular weight fraction with biological activity.

When administered iv, MVE-2 is taken up by and localized in the monocyte–macrophage RES compartment, and 20% of the activity of an injected dose persists at 5 weeks. The immune augmenting action is independent of IF induction, although this does occur and is most closely related to long-lasting macrophage and NK cell activation. Toxicity, side effects, and untoward reactions to be expected would include hepatosplenomegaly; inhibition of hepatic mixed function oxidases; heparin-like anticoagulant activity; potential activation of suppressor T-cells; and clinically, fever, chills, and impaired liver or renal function. The above expectation is based on observations of pyran.

We recommend that 3 independent groups conduct the phase I trials with this agent in patients with advanced, chemotherapy refractory malignant disease of known limited life expectancy, who are, however, in satisfactory clinical condition so that they are likely to respond to biological stimulation. Thus patients with severe immunodeficiency would not be candidates.

Clinical monitoring should include acute studies of cardiovascular status and serial studies of pulmonary, hepatic, renal function, and hematological function. Researchers should attempt to study the pharmacology of the agent with radiolabeled compounds which will be available from Adria Laboratories. Host defense monitoring is mandatory. This should include pretreatment evaluation of general immunocompetence with delayed hypersensitivity, leukocyte subpopulation enumeration, and lymphocyte blastogenesis. Before and during treatment, monitoring for biological response modification should include measurement of IF induction, NK cells, ADCC, monocyte-mediated cytotoxicity, monocyte precursor numbers in the peripheral blood, serum lysozyme, and if possible, RES clearance of particulate matter. Evidence for optimal biological response modification will be the data base on which phase II–III clinical trials will be developed.

The phase I studies of MVE-2 should establish a rational clinical approach to the evaluation and development of other augmenting agents. With the establishment of this mechanism and later in the Program, phase I studies should be possible of other promising augmenting agents such as MVE-r, poly I:C with mismatched bases, lipoidal amines, NED137, low molecular weight IF inducers, glucan, and other polysaccarides, etc. These compounds would be introduced into the program according to established channels.

Further Development of Chemoprevention

The program components suggested for initiation of a treatment effort with retinoids are discussed under the same general headings as the other BRM, but the emphasis and needs for chemoprevention are unique. The clinical trial components focus on "patients" without manifest disease; the basic research and development aspects must interface and depend on programs already operative in other Divisions (Cancer Cause and Prevention and Carcinogenesis) and when production of current or future agents is advanced in the private sector and well-funded through NCI. The BRM Program emphasis should be placed on careful interpretation of preventive agents in therapy regimens in which risk is predictably high. Modeling of treatment combinations before large-scale trials can be opened may be necessary.

Clinical Trials

Selection of patients for clinical trials with retinoids is a fundamentally unique problem, with criteria different from those used in the selection of patients for therapy trials. Because of the nature of the activity of retinoids, patients suitable for chemoprevention protocols must be free of disease. Response can be measured in time to new lesions, and whether they are de novo, recurrent, or second primaries.

The time necessary for assessment of significant delay, as well as for dose intervals, may be long and thereby may present additional problems in design of these clinical studies. (Lifetime studies might be required.) There are high-risk patient categories in which trials may be done after appropriate phase I biological response trials are evaluated.

Subjects to be included in eventual efficacy trials for prevention will be "precancer" or cancer patients with no evidence of disease. Subjects for the careful toxicity and biological response initial trials should be as biologically normal as possible; patients with multiple senile keratoses, psoriasis, or severe acne may be available for detailed evaluation.

The phase I studies should include frequent assessment of kinetics and quantitative changes in relevant host parameters. These should include hematological and liver functions, lymphoid functions including T-cell activities, and surface properties of lymphocytes (HLA or Iq). Concentrations of drug in blood, urine, and biopsy material when available should also be measured. Specialized assays for T-lymphocyte colonies, CFU, skin fibroblast growth characteristics, or rates of PBL mutagenesis may also be important. Inasmuch as phase II studies may include patients who have been or are simultaneously on chemotherapy, additional phase I trials are needed for evaluation of biological effects of chemopreventives in patients also receiving chemotherapy. For example, retinoids may affect the T-cell suppressive marrow or epithelial toxicities, or mutagenicity of chemotherapy.

Although certain therapeutic chemopreventive trials have been initiated previously and efficacy trials for high-risk patient groups should be instituted at an appropriate time, they are not recommended now. It is essential that preclinical studies be supported so that therapy interactions, tumor recurrence, and reversal of mutagenicity can be modeled.

Preclinical Research and Development

The development of new retinoids with improved therapeutic indexes is of high priority to this Program, in particular the development of compounds with well-defined organ specificity. Such specificity, as apparently demonstrated for 4-hydroxyphenyl retinamide and the rat mammary gland, may be most important in the limitation

of the extent of any drug-induced toxicity. Synthesis of retinoids is already well funded under the Division of Cancer Cause and Prevention/NCI contract mechanisms; research in this area should be conducted in close collaboration with this Division.

Production of Tumor Necrosis Factor

The serum of BCG-infected mice treated with endotoxin contains a substance (TNF) that mimics the tumor-necrotizing action of endotoxin itself. This factor, not a residual endotoxin, is released from host cells, probably macrophages. The TNF induced in the same way in rats and rabbits also causes necrosis of transplanted murine tumors. Unlike endotoxin, TNF is toxic in vitro for neoplastic murine and human cell lines but not for mouse embryo culture. It has striking effects on immunological reactions in vitro, some like and other unlike those of endotoxin. Highly purified preparations of TNF, a glycoprotein with a molecular weight less than 70,000, do not contain lysosomal or nonlysosomal serum enzymes, IF, or PGE_1. Some scientists have proposed that partially purified murine TNF should be produced for further study in experimental animals and for initial clinical evaluation.

Mice are first given injections of *C. parvum*, and 9 or 10 days later they receive endotoxin. Blood is collected 1.5 hours later. Serum is separated by centrifugation, and then batches of serum are pooled and tested in vivo and in vitro for TNF activity.

In a 20-g mouse, a single injection of 100 μg of partially purified TNF induces necrosis of a 1-g cutaneous transplant of sarcoma Meth A, given iv as well as by the intratumoral route. Because the ratio of body weight to tumor weight (and thus the dilution after systemic administration) in a 70-kg patient with a 1-g cutaneous metastasis is 2,500 times greater than in the mouse, initial evaluation by direct injection into cutaneous metastases is proposed. The objective will be determination of whether single or multiple intralesional injections of TNF induce necrosis and regression of injected lesions.

Mouse serum may contain lymphocytic choriomeningitis virus and Sendai virus. In rare instances, these viruses have caused mild self-limited upper respiratory infections in man. Even if the serum contained these viruses at the start, it is highly unlikely that they were not eliminated by the purification procedure which includes ultracentrifugation and chromatography. Partially purified TNF is not pyrogen free. However, because a dose of 100 μg will fail to exceed the pyrogenic equivalent of 0.002 μg endotoxin, fever is not likely to occur at that dose. Beginning at that starting point, tolerance of higher doses can be safely determined.

Definition of Distinctive Cell Surface Antigens of Human Cancers and Development of Tumor-specific Immunotherapy

One biological response of the cancer patient that might be modified to therapeutic advantage is the immune response to cancer-restricted cell surface antigens (*see* Section VI). Two basic approaches by which augmentation of the immune response to cancer antigens can be induced are nonspecific immunopotentiation (which constitutes the bulk of past and current efforts at cancer immunotherapy) and specific immunization (which has not been evaluated in the treatment of cancer but has contributed much to the control of infectious diseases). The knowledge of microbial antigens has permitted the development of successful specific immunization against infections and relegated nonspecific immunostimulants to an adjuvant role. Lack of knowledge of human cancer antigens, on the other hand, has long prevented exploration of specific immunization in the context of cancer as it should be explored, i.e., with the use of preparations with defined cancer-restricted antigenicity and demonstration of their immunogenicity in cancer patients. It is our contention that progress in the definition of human cancer cell surface antigens now permits investigation of specific immunization and that this immunization should be a component of the BRM Program from the start. The 3 approaches that can be envisaged are: 1) active immunization with immunogeneic preparations of cancer-restricted antigens, 2) passive transfer of immunity with specific antibody (the antibody being expected to cause complement-mediated cancer cell destruction) or the use of specific antibody conjugated with cytotoxic drugs or radioisotopes for cancer cell-restricted delivery of these agents, and 3) adoptive transfer of immunity with specifically sensitized lymphocytes. Although passive transfer of immunity may become feasible with hybridoma-produced monoclonal antibodies in the near future (*see* Section IX), more developmental work is required before this approach to therapy is ready for clinical testing. Similarly, adoptive transfer of immunity with lymphocytes (*see* Sections VII and IX) could become a realistic possibility as a consequence of the discovery of the TCGF which permits propagation of specifically sensitized T-cell clones in vitro. Again, more experimental research is required before this approach can be tested in the clinical situation. Specific active immunization can be tested clinically based on the accomplished definition and classification of cell surface antigens of some human cancers, such as malignant melanomas, renal cell cancers, and astrocytomas.

By autologous serological typing, three classes of cell surface antigens have been identified (*see* Section VI). Class 1 antigens are restricted to autologous tumor cells and cannot be detected by absorption tests on normal or malignant cells of any other type, autologous, allogeneic, or xenogeneic. Class 2 antigens are found on autologous as well as certain allogeneic tumors but cannot be detected on normal cells. On the basis of current evidence, Class 1 and Class 2 antigens can be considered tumor-restricted antigens capable of eliciting humoral immunity in the autologous host. In contrast, Class 3 antigens are a diverse and complex group, with some showing differentiation characteristics, some alloantigenic characteristics, and others heterospecific characteristics. The BRM Program should support the construction and clinical testing of various types of cancer antigen preparations with the long-term objective of developing maximally immunogenic preparations from types of cancer, the cell surface antigens of which have been defined serologically.

Even under the most appropriate circumstances and with a most carefully designed study, the testing of human cancer antigen preparations is fraught with difficulties and uncertainties ranging from appropriate type, dose, and route to suitable patient selection (type and stage of cancer) to a critical evaluation of clinical response (tumor reduction, tumor recurrence, survival time). Given this array of variables, a sequential analysis of the clinical effectiveness of different preparations in various patient populations would, by an evaluation of clinical response alone, be extremely time-consuming and expensive and possibly not result in a picture any clearer with regard to the value of this approach than has emerged from past studies. What is required for the development of immunogenic cancer antigen preparations are methods by which effectiveness can be assessed rapidly and objectively and that can be used to guide the step-by-step process of vaccine construction and testing. With regard to vaccines against infectious diseases, serological responses to bacterial or viral antigens have been an essential step in their development. Our lack of comparable serological tests to monitor the effectiveness of cancer vaccines in humans has been a major impediment to investigating this approach to cancer therapy. With the development of autologous and allogeneic typing systems for defining cell surface antigens of melanoma and other cancers, we can now use serological tests of requisite sensitivity and specificity to gauge the immunogenicity of cancer vaccines. With these methods, the immunogenicity

TABLE 1-2.—*List of projects to be developed initially within the BRM Program*

1. In vitro monitoring of correlates of antitumor activity of BRM in humans
2. Study of BRM in combination with antitumor agents and study of combined modalities
3. Correlation of direct measurements of IF and thymic hormone levels in blood, body fluids, and tissues with antitumor and other biological response modification in humans
4. Study of thymic polypeptides and factors for selectivity of action on tumor and immune balance
5. Studies of the mode of action of thymic factors
6. Studies of the cellular and molecular bases for the antitumor effect of IF and comparison of antitumor vs. antiviral activity
7. Studies of the tissue specificity of IF
8. Studies of the immunomodulatory effects of IF and effects on cell–cell interactions
9. Study of IF inducers and modification of induction
10. Development of reference Type II IF (mouse and human)
11. Identification, isolation, and purification of IF and active fragments and their testing in vivo
12. Definition of human cancer antigens: serology and immune reaction (including monoclonal antibodies)
13. Purification and modification of cancer antigens: preparation and evaluation of specific cancer vaccines
14. Immunotherapy with monoclonal antibody
15. Adoptive immunotherapy by lymphoid and nonlymphoid cells
16. Identification of effector cells in adoptive immunotherapy
17. Use of continuous lines or clones of T-cells for immunotherapy
18. Selective modification of suppressor cell function for immunotherapy
19. Modulation by BRM of cellular antitumor responses in vitro and of therapy in vivo
20. Study of the modulation of macrophage function and definition and measurements of these functions
21. Mechanisms of escape from tumor immunity
22. Immunoregulatory role of prostaglandins and prostaglandin inhibitors
23. Definition of mechanisms of specific and nonspecific immunosuppression in cancer patients and investigations of methods of abrogating immunosuppression
24. Development of hybridomas for monoclonal antibodies against appropriate BRM
25. Study of purified microbial adjuvant subcomponents
26. Effects of retinoids on immune functions; comparisons of natural and synthetic retinoids and interactions with other BRM
27. Studies of mechanisms of action of retinoids at the cellular and biochemical levels
28. Pharmacokinetic and drug distribution studies with retinoids
29. Study of antileukemic plasma factors
30. Plasmapheresis and selective plasma component depletion in cancer therapy
31. Lymphokines: production, purification, and study of in vivo antitumor effects
32. Design of new therapies based on specific responsiveness of tumor cell populations to growth factors
33. Design of new therapies based on knowledge of the role of tumor growth-promoting factors in vivo
34. Utilization of TCGF and other specific cell growth factors as BRM
35. Regulation of tumor cell populations by normal cells or cell products
36. Use of growth and differentiation factors in the protection and/or restoration of normal tissues in connection with the toxicities of anticancer therapy
37. Studies of the effects of chemotherapy and radiotherapy on biological responses
38. Study of the effect of BRM on metastatic tumor cells and sequestered cells in animal models
39. Studies of the effects of nutritional factors on BRM activity
40. Study of delivery and targeting systems for BRM
41. Study of the BRM modulation by nonantineoplastic agents
42. Studies of the pharmacological regulators of immune response and of host-tumor interactions
43. Purification and development of RIA for promising BRM

of different cancer antigen preparations should be tested in series of 10–15 patients each.

Development of Systems for Evaluation of Biological Response Modifiers

The need for the development of test systems and biological models for implementation of the BRM Program is indicated in various parts of this Report. These include such defined systems as the development of suitable tumor models for common track evaluation in step 5 of the Network and of cancer prevention tests, or the numerous studies required in many areas of BRM for the optimal implementation of the Program; these range from the refinement of clinical monitoring tests to the development of improved methods of purification or formulation of agents, or both.

Overall Preclinical Research Program

A major research program should be developed in the area of BRM that has fundamental implications toward: 1) the development of new leads through the understanding of the phenomena involved in the actions of known BRM; 2) the improved utilization of known BRM and the identification of new therapies involving these agents; 3) the improved production of certain BRM and the identification of defined BRM chemical structures which may become the target of chemical synthesis. Part of this research program should be implemented through contracts, part through grants awarded in response to RFA, and part as a result of the stimulation of the scientific community through program announcements which would encourage fully investigator-initiated and proposed research. Some of the items that might be included in this research program can be found in various parts of this Report. A number of items that are suggested as subjects of initial Program implementation are listed in table 1-2 and have been formally submitted to the Division of Cancer Treatment Board of Scientific Counselors as part of the Subcommittee's recommendations.

SECTION II

Clinical Aspects of Biological Response Modifiers:

Current Status and Future Prospects

PREAMBLE

Introduction

Clinical therapeutic investigations of BRM, almost exclusively with crude microbial adjuvants such as BCG, *C. parvum,* MER, have been conducted by many scientists over the last 10 years. Most of the major types of human cancers have been treated with the agents, either alone or as an adjunct to surgery or chemotherapy. Some of these trials have indicated that immunotherapy can in itself induce regressions of local lesions (melanoma) or distant metastases (renal cancer), and some have simply shown that immunotherapy can prolong remission duration and/or survival induced by conventional therapy. An important new element has been the demonstration that IF, a product of stimulated normal human cells, can induce tumor regression in a fraction of patients with malignant lymphoma, multiple myeloma, and breast cancer.

The studies of immunotherapy conducted to date must be considered only to be indicators for further research and are not definitive proof of activity or indicators that any of these modalities are the treatment of choice for human neoplasms. The basis for this reservation is that the trials are controversial, and preliminary observations have not regularly been confirmed by other investigators. Often the studies were 1) not adequately controlled for comparability of prognostic variables in treatment and control arms, 2) not done with large enough numbers of patients for adequate statistical analysis that would help one detect differences even as large as 30%, 3) reported prematurely, and 4) almost none were done with measurements that would show biological responses were indeed modified.

Despite these reservations, the area of BRM therapy research is of high priority because of the suggestive data from the clinical work thus far, the recent results with IF, the intrinsic logic of the approaches in utilizing or augmenting "natural" defense mechanisms, and the recent advances in both the production of more defined therapeutic agents and in potentially applicable monitoring techniques.

Within this historical background and conceptual framework, we have attempted to develop a rationale and scheme for development research on BRM in the clinical setting. The basis for this has been a critical review of the clinical trials in the various human malignant disease categories, a detailed and critical review of the clinical trials oriented to the class of therapeutic agent, and a critical perspective of the therapeutic agents and their potential for refinement, development, etc.

These reviews in turn have led to the development of specific proposals for a mechanism by which a new potentially therapeutic agent can be developed for clinical trial, a scheme for relevant biological response monitoring and priorities both for agent selection and confirmatory trial conduct. It is hoped that this scheme will bring not only new agents but a new therapeutic modality into a major role in the management of cancer.

A number of large-scale confirmatory trials of various modalities of immunotherapy combined with conventional therapy, underway at the present time, mainly involve the use of intact and crude extracts of microorganisms. They may be classified as first generation immunotherapy agents.

We must now move on to second generation immunotherapeutic agents. These are defined as synthetic agents or agents which are purified extracts from crude tissues or intact microorganisms, as agents whose specific mechanism of action has been studied and is partially understood, and as agents which have potent effects on host defense mechanisms. These second generation agents may be those that can be administered systemically with less toxicity than some of the previously used crude microbial adjuvants.

No major program effort is recommended for most of the first generation agents. However, individual proposals involving first generation agents and phase II and III trials of these agents by individual institutions or groups must be carefully considered. It is likely that certain specific uses of first generation agents will ultimately enter the therapeutic armamentarium against cancer.

The objectives of the program proposal in broad terms are the performance of basic, preclinical, and phase I–II clinical studies of second generation immunotherapeutic agents. These studies should be done in defined, preclinical, and clinical models for which guidelines will be provided as outlined below. In addition, the development of the program will depend greatly on dose–response studies and on in-depth immunological and biological monitoring of the subjects receiving therapy. It will be essential that this component be done so that rational doses and schedules of treatment can be selected for trials. Selection of categories of agents and histological types of malignant disease to be treated will be based on preclinical and clinical results, an in-depth understanding of the biology of the disease and the biological activity of the agent, and on evidence of potent host-modifying activity of the agent.

Immunotherapy trials to date often included careful attempts toward immune profiling or other evaluation of biological functions and correlation of these results with those obtained clinically. No skin tests, lymphocyte response, or other measure satisfactorily quantified the complex host response to the tumor. However, our ability to monitor and analyze host defense mechanisms and other biological functions and to evaluate the modulation induced by therapy has also reached the second generation. Thus we can now evaluate and monitor in detail T-cell subset functions (helper, killer, suppressor), other suppressor functions, a spectrum of leukocyte subsets based on their surface markers, monocyte and macrophage functions in vitro, RES function in vivo, a wide variety of serological factors, IF induction, circulating levels of thymic hormones, etc. Most of these techniques have been developed during the last 5 years, and expertise in these methods puts us in a much better position to evaluate new agents and to re-evaluate agents studied in the past.

For example, almost all preclinical and all clinical studies utilizing active nonspecific immunotherapy with agents, such as BCG and *C. parvum,* have been done without an evaluation of suppressor cell function and its modulation by therapy. Considerable recent data indicate that suppressor cells may be activated by the systemic administration of these immunotherapeutic agents; this

must be taken into account in this type of therapy. It also suggests that combined active nonspecific immunotherapy and antisuppressor cell therapy may greatly augment the activity of the active nonspecific immunotherapy.

Prior to broad phase II and III clinical trials, certain criteria should be met. These include the demonstration of measurable, systemic biological effects and the demonstration of dose–response relationships from phase I studies. When possible, optimal immunological or biological modulating dose, route, and schedule must be determined before moving to phase II trials. Monitoring should be possible with assays as relevant as possible to the particular immunotherapeutic or biological response modifying agents. *No longer acceptable are the large phase II and III clinical trials with doses and schedules or routes of administration of treatment that have been selected arbitrarily without some evidence for biological activity, dose optimization, and monitoring feasibility.* An exception to this approach would be the confirmation or extension of studies of therapies of previously demonstrated clinical utility.

Preclinical Therapeutic Evaluation of Biological Response Modifiers

The recommendation for clinical trials should be based on a tumor-bearing animal model. The agent must have antitumor activity in that model which may be manifested by tumor regression or an adjuvant activity in prolongation of survival after conventional treatment. Some of the characteristics of the model should include: 1) antigenicity or lack thereof of the tumor has been defined; 2) tumor genetic relationship to the host is also defined; 3) the model is a true therapy model (in other words, the tumor must be in place and growing either macroscopically or microscopically when the treatment is started; therapy prior to or simultaneous with tumor inoculation is not acceptable); 4) biological modification of the host must be demonstrable; and 5) monitoring of host modification in the model should be translatable into related host monitoring in man. A broad spectrum of models as proposed by investigators should be welcomed.

The agents to be studied must be biologically characterizable as modulating a host defense or other biological phenomenon in the model, and host modulation must be monitorable similarly in man. Alternatively, an agent can be recommended for clinical trials if it has demonstrable host response-modifying activity such that one can logically assume it may have therapeutic activity in human cancer known to be associated with immunological abnormalities. This assumption would be particularly relevant when those host abnormalities are not commonly demonstrable in animal models. An example would be thymic hormone immunotherapy. Furthermore, biological agents which have documented antitumor effects but which cannot be demonstrated to modify a known biological response should receive individual consideration. Finally, models of immunodeficiency or immune deviation that mimic defined clinical entities in man should be encouraged.

Clinical Model and a Phase I–III Development Scheme for Systemic Biological Response Modifier Therapy

Objectives

To conduct defined phase I–III studies of BRM, investigators should determine definite toxicity at the MTD, demonstrate host defense modification or other biological effects, select optimal dose and schedule relevant to the biological effects (the optimal dose and MTD may differ), predict interactive variables for combination of conventional with experimental therapy, and conduct therapeutic trials.

Patient Selection

Clinically appropriate patients should be selected so they will be reasonable approximations of the ultimate patient groups to be investigated in phase II–III trials. For toxicity studies, patients must have known limited life-span and must have received all drugs of higher priority; in other words, all more appropriate forms of therapy must have been given. For biological response and optimal dose study, patients must be relevant as to the above but may have disease of any stage. Preference will be given to studies including stable patients not on other therapies that could complicate interpretation of test results. It would be preferred, but not mandatory, that the studies be done in clinically homogeneous groups according to histology and stage of disease so that variability in biological responsiveness between and among patients is minimized. If an agent comes to light with proven antitumor activity, it can be introduced into phase II trials as long as dose–response and toxicity studies are also supported and conducted.

Controls

Appropriate controls are central to the development scheme. Effects of immunological manipulations must be studied relative to appropriate controls, in regard to the patient group, the agent, and the schedule of therapy. Consideration must be given in the selection of controls to the natural fluctuations in host reactivity observed in normal and diseased human subjects. Experimental design must be based on detailed knowledge of these fluctuations relevant to a particular test.

Types of Studies

These will include those on single, escalating, and chronic doses, with the latter dosage used in the phase II–III trials.

Outline for Study of Therapeutic Efficacy

For agents which themselves cause tumor regression, i.e., activity in reducing the size of measurable tumor, the classical phase II design, as in chemotherapy trials, is appropriate.

For agents which do not have direct antitumor activity, but which serve as adjuvants to conventional therapy, special efficacy trials need to be designed. These trials must be appropriately controlled with groups not receiving biological therapy and should include monitoring so the induction of the desired biological effect is assured. Study conclusions must await firm data rather than statistical projections based on preliminary results. In those circum-

stances when the dose of therapy must be modulated, serial monitoring is mandatory. The trial must have an appropriate experimental design and an adequate number of patients. In the development of a clinical phase II–III study of a BRM, emphasis must be given to the biological status of the tumor-bearing host, the effects of conventional therapy on that status, and how the BRM will effect this status.

Site/type of cancer	
Lung	Renal
Breast	Head and neck
Gastrointestinal	Malignant melanoma and other skin cancers
Gynecological	Leukemia
Bladder	Lymphoma

Current classes of agents of interest
Microbial agents
Intact organisms or crude extracts
Purified fractions
Cellular products
Thymic hormones
IF
Synthetic agents
Immunostimulators
Immunorestoratives

CLINICAL TRIALS REVIEW

Lung Cancer Immunotherapy

Introduction

Immunotherapy of lung cancer has involved the use of active nonspecific immunotherapy with agents, such as BCG, MER, and *C. parvum;* presumed active specific immunotherapy with tumor extract mixed with complete Freund's adjuvant; and immunorestorative immunotherapy with such agents as levamisole and thymosin. Routes of administration have included cutaneous, intralesional and systemic, or iv therapy. Most studies have involved adjuvant therapy after definitive surgery for stage I disease, but a variety of studies have been done on the immunotherapy of patients with stages I–III or IV disease. Although well over 50 reports have been published since 1970 on the immunotherapy of lung cancer, few have been definitive and have yielded data which can be interpreted without equivocation.

Lung cancer has been selected with high priority for immunotherapy for a number of valid reasons. 1) It is 1 of the 3 most common malignant neoplasms in the American population. 2) The overall 5-year survival is less than 10%. Even in those patients in whom stage I disease exists and the tumor can be completely removed surgically, the 5-year survival is only approximately 35%. 3) The chemotherapy for all forms of lung cancer, except oat cell carcinoma, is most unsatisfactory with remission rates of less than 50% and remission duration of less than 1 year. 4) Immunological studies of patients with lung cancer have revealed that often a profound immunodeficiency is characterized by reduced numbers of T-lymphocytes, reduced T-lymphocyte functions such as blastogenic responses, diminished lymphocyte-mediated cytotoxicity, and diminished in vitro delayed hypersensitivity. Also, a relationship between immunocompetence and prognosis exists in this disease, with competent patients showing a better prognosis, which suggests that immunorestorative approaches to therapy are beneficial.

In this review, we will examine 7 approaches to immunotherapy of lung cancer in which definitive studies have been done or are in progress. The overall impression from these data is that immunotherapy can significantly prolong remission duration and/or survival induced by conventional therapy in this disease, but we have no evidence that immunotherapy increases the cure rate.

Intrapleural Bacillus Calmette-Guérin

This work was pioneered by McKneally at the Albany Medical College (*1*) and was based on the observation that patients with empyema after definitive surgery for lung cancer had an improved survival, compared with those who did not experience this infectious complication. McKneally and his colleagues speculated that this was due to activation of host defense mechanisms by the infectious process and that BCG, a microbial immunostimulator and adjuvant, would be effective in this regard. They did preliminary studies in animal species to demonstrate the safety of this approach. Subsequently the clinical trial was designed.

In the initial report of this work (*1*), 60 patients were randomized after surgical documentation of stage of disease (according to the criteria of Mountain et al.) to receive a single intrapleural injection of 10^7 viable U of Tice strain BCG. They were also treated with repeated doses of aspirin and Benadryl for 48 hours after the injection. Fourteen days after the injection, patients and controls were treated with 300 mg INH/day for 12 weeks. The controls did not receive a placebo injection intrapleurally. Patients were followed at 2, 4, 12, 26, and 52 weeks and every 6 months thereafter by chest X-ray. They were also stratified by histological types of tumor into 3 groups: anaplastic large cell carcinoma, adenocarcinoma, and squamous cell carcinoma. Oat cell or small cell anaplastic cancer was excluded.

No significant short-term effect of therapy was observed in patients with stages II and III disease. However, longer follow-up may reveal an effect. In the initial report, 17 BCG-treated and 22 control patients had stage I disease.[1] They were reasonably well balanced according to various prognostic criteria, except the controls had fewer patients over the age of 60 years and more patients operated on the left side. At the time of the initial report in 1976, with a median follow-up of 12 months, 9 or 41% of the controls experienced relapses, whereas no one in the BCG group

[1] Stage I disease is defined as a single parenchymal lesion under 3 cm in diameter without chest wall or pleural involvement or spread to mediastinal lymph nodes.

did. More patients have been added and, at the present time, they have 30 treated patients and 36 controls with stage I disease. Eleven relapses (9 deaths) occurred in the treated patients and 23 (15 deaths) in the controls. The median survival is approximately 24 and 52 months in the controls and treated patients, respectively. The difference is highly significant with a *P* value of 0.0066. Importantly, there does not seem to be a plateau phase on the survival curve of either the treated or the controls yet, and it cannot be determined whether the patients who are treated have an increased long-term survival, i.e., a cure (McKneally MF: Personal communication).

This study might be criticized because of the poor survival of the controls compared with studies in other institutions. However, its validity is strengthened because this is a prospectively randomized trial. One must assume that the treated patients would have done as poorly as the controls had they not been treated.

In late 1977, another investigation of postsurgical immunotherapy was started by McKneally and co-workers in which repeated BCG scarifications were added to the postsurgical intrapleural BCG. Insufficient time has elapsed for evaluation of the results.

Several attempts by other scientists are currently underway to confirm the observation of McKneally and associates. One is being conducted by Hill and Wright and their co-workers at The Fred Hutchinson Cancer Research Center for the Northwest Oncology Group (*2*). This is a 3-arm study in which patients are randomized to receive intrapleural placebo, intrapleural BCG exactly as given by McKneally, or BCG plus levamisole at 100 mg/m^2 of body surface area daily for 2 days repeated every 2 weeks. Hill et al. are still accumulating patients. Currently, 34 patients are in the placebo arm, 47 on BCG, and 55 on BCG plus levamisole. Patients with stage I disease can be evaluated by this regimen, defined as above, although patients with stages II and III resectable disease are also being treated. Currently, the median follow-up is approximately 18 months. There have been 31% recurrences in the placebo group, 27% in the BCG group, and 38% in the BCG plus levamisole group. The median remission duration in the control groups is estimated to be approximately 2 years, in the BCG group it has not been reached, and in the BCG plus levamisole group, it is estimated to be 18 months. None of the differences are significant, although apparently the BCG plus levamisole group is doing slightly worse. The difference in the current results of this study and the early ones of McKneally et al. indicate the major differences that can occur between different clinics and the need for multiple confirmation of a potential positive result. More stage I patient entries and longer follow-up will be needed for evaluation of this study.

Another repeat evaluation of the McKneally trial is being conducted by the Lung Cancer Study Group composed of scientists at the Mayo Clinic, M. D. Anderson Hospital and Tumor Institute, University of Washington at Seattle, Princess Margaret Hospital at Toronto, University of California-Los Angeles, and Vanderbilt University (*3*). Thus far, 264 patients with stage I disease, defined as above, can be evaluated on a protocol to receive BCG followed by INH as done by McKneally, or to receive both BCG and INH placebos. The median follow-up is 259 days. The 39 recurrences were evenly divided between the 2 groups; also, no significant difference in survival was noted between the 2 groups. This study will probably be closed. Several other groups, including the Southwest Oncology Group, are currently evaluating this or related approaches, and data should be available within the next 2 years.

Problems with these studies include differences in staging criteria and in relapse rates in the control groups and the addition of a third arm to the study (that increases the variance of the estimate). One must avoid premature positive or negative conclusions, particularly to preclude cessation of patient accrual.

Cutaneous Bacillus Calmette-Guérin *or Its Methanol Extraction Residue*

Among the several studies of the use of BCG administered by the cutaneous route in lung cancer, Pines (*4*) treated patients with advanced regional squamous cell lung cancer with local radiotherapy and a 14-day course of 200 mg CPP iv daily. The patients were randomized to receive 25–120 million Glaxo organisms by Heaf gun weekly for the first year and then monthly: Twenty-five patients received BCG, 23 were controls. The difference in survival was significant during the first year, with 5 deaths among the BCG patients and 12 among the controls. The incidence of distant metastases among the controls was also significantly higher, with 12 cases or 52% versus 2 cases or 9%. However, at the end of 5 years of follow-up, the survival rate was almost the same; 22 of the patients and all the controls died.

In another study, Mathé and co-workers (*5*) treated patients with stages I and II squamous cell carcinoma with 75 mg Pasteur BCG by scarification weekly for 1 year and then every 2 weeks after definitive surgery. Thirty-nine patients were randomized into the BCG or control groups. With a median follow-up of 2 years, survival was significantly improved among the treated patients. Because it is too early in this study to determine if long-term survival will be improved by BCG, one could not predict improvement on the basis of the Pines' study because of the differences in the BCG product used, the dose, method of administration, stage of disease, histological type of disease, and associated therapies.

Edwards and Whitwell (*6*) randomized patients to receive a single dose of Glaxo BCG (500,000 organisms sc 10 days after surgery for stages I and II disease). The patients were well balanced according to prognostic variables including histological type, presence of positive lymph nodes, etc. In their initial report published in 1974, the trend toward improved survival for the BCG-treated patients was significant for the overall group and for several subgroups. The overall survival at 2 years was 38% in the controls and 52% in the BCG-treated patients. In a subsequent follow-up after 5 years, the difference between the 2 groups of 60 patients was not significant (*7*): It was 20% in the 30 controls and 30% in the 30 patients treated with BCG. In the subgroup who underwent lobectomy, survival was 25% in the controls and 39% in the BCG group, but because of the small numbers (11 and 17 patients, respectively), this was not statistically significant.

The combined data for these 3 studies do suggest a limited but measurable effect of BCG immunotherapy in lung cancer when it is given percutaneously. This approach warrants further definitive study with more potent adjuvants in larger numbers of patients.

An example of other studies on BCG-related materials in lung cancer is that of Robinson et al. (*8*) who found no significant difference between a small heterogeneous group of lung cancer patients treated with surgery, radiotherapy, and/or chemotherapy, and a similar group treated with the same plus intradermal MER. However, some immunological boosting of skin tests and blastogenesis was seen in the MER-treated patients. The major criticism of this study is that the groups were not well balanced for important clinical features and, therefore, the study is difficult, if not impossible, to interpret. Many of the randomized immunotherapy trials in man are not definitive because of these limitations.

Overall, the major problem with these investigations of cutaneous BCG or MER is the small numbers of patients involved. Small but important differences in remission duration and survival will be missed (nonsignificant) due to this deficiency. In addition, with adjuvants of limited activity, such as BCG, only a temporary effect is achieved. This may be improved by the use of more potent agents, different routes of therapy, or more prolonged therapy. Also trial design, adequate stratification of patients, and adequate number of patients will be essential.

Primary Intratumor Bacillus Calmette-Guérin

In addition to the above research with BCG, given intrapleurally or cutaneously, 2 important preliminary studies with BCG given intralesionally are those of Holmes and co-workers (*9*) and Matthay et al. (*10*), who are studying the feasibility of administering BCG through a long needle directly into the primary stage I lung cancer, allowing an immunological reaction to proceed for 2 weeks, and then removing the primary tumor. This is based on observations in the guinea pig hepatoma model of Rapp in which this form of primary immunotherapy results in cures of animals with regional metastatic disease. The procedure has been found to be safe, to induce an inflammatory reaction in the primary tumor and some tumor regression or necrosis, and is now in phase II clinical trials. Definitive data are not available.

Corynebacterium parvum *(Subcutaneous and Intravenous)*

Immunotherapy with *C. parvum* in patients with lung cancer has also been investigated fairly extensively. Studies have been conducted by various individuals and are also being conducted by some of the cooperative groups in this country. In general, they show prolongation of surgical- or chemotherapy-induced remissions and survival of the treated patients compared with randomized controls.

One of the earliest workers in this field was Israël who in 1974 reported randomized studies in metastatic solid tumors including lung cancer treated either with chemotherapy (CPP, MTX, 5-FU, procarbazine, and vincristine) or chemotherapy plus sc *C. parvum* (*11*). Three studies were done with approximately 140 patients randomized into 2 groups. The prognostic variables for the patient groups were not well characterized in the reports. The *C. parvum* was administered at a dose of 4 mg sc weekly; the median survival was approximately doubled by this therapy. Thus in the first study at 6 months, 32% of the controls survived as did 59% of the treated patients. In the second study with patients having bronchogenic squamous cell carcinoma, the median survival was 4 and 9 months for controls and treated patients, respectively. In the third, 40 patients were randomized, and the mean survival was 9.1 months with *C. parvum* and 5 months without. These differences were highly significant statistically.

Dimitrov and co-workers (*12*) randomized 65 patients with stage III bronchogenic carcinoma to chemotherapy with adriamycin or chemotherapy with adriamycin plus immunotherapy with *C. parvum* given weekly as multiple sc injections. The response rates were similar in the 2 groups. However, patients treated with adriamycin and *C. parvum* had a longer survival (11 vs. 7 mo). The difference was at the borderline of significance.

More recently, Takita and his group [Bjornsson S; (*13*)] investigated the use of *C. parvum* immunotherapy in lung cancer. In one study, 76 patients with stage III bronchogenic carcinoma were randomized to receive combination chemotherapy or the same plus monthly iv *C. parvum* at a dose of 0.25 mg escalating to 3 mg or to receive the chemotherapy with MER intradermally. Survival among patients receiving the *C. parvum* was significantly longer than that of those receiving either MER or no immunotherapy. The median survival was 20 weeks in the chemotherapy group, 19 in the group receiving MER, and 28 in the *C. parvum* group. This study certainly warrants extension and confirmation. In the other study, 32 patients with inoperable lung carcinoma (limited disease), excluding the small cell type, were randomized to receive chemotherapy with Me-CCNU and vinblastine versus the same with iv *C. parvum* as outlined above (*14*). Of the patients who received an average 1.8 courses of chemotherapy (controls), 1 entered remission and 12 died; of those in the immunotherapy group who received an average of 2.8 courses of chemotherapy, 4 entered remission and 6 died. This study also needs confirmation and extension.

Thus in summary, *C. parvum* given sc and iv has a modest but statistically significant effect on remission duration and survival in non-oat cell carcinoma of the lung. Not every study has been positive. Should anyone conduct confirmatory field trials, they will require large numbers of patients to assure that statistically valid conclusions can be drawn on small differences between groups.

Tumor Extract Mixed With Complete Freund's Adjuvant

Another approach to immunotherapy in lung cancer which has been claimed to show promising results has been the administration of tumor antigen extract combined with complete Freund's adjuvant. In the first study, which was conducted by Stewart and Hollinshead and their coworkers (*15*), 55 patients with stages I and II resectable, non-oat cell carcinoma of the lung were randomized for therapy at the time of curative surgery. Three arms of treatment were studied. High-dose MTX followed by citrovorum factor rescue once a month for 3 months;

immunization once a month for 3 months with 300–500 g of tumor antigen homogenized with 1 ml of complete Freund's adjuvant monthly for 3 months; and a third group received a combination of this therapy with immunization administered 7 days following chemotherapy for 3 cycles. In addition, a group of 16 concomitant stage I controls from another hospital were also followed. Thus 26 patients were in the MTX and control groups, 16 in the immunotherapy group, and 13 in the immunochemotherapy group. The numbers of patients in these groups with stage I disease were 24, 13, and 11, respectively. Six deaths occurred in the total combined control group, none in the immunotherapy group, and 1 in the immunochemotherapy group as of the fall of 1977. In an update on the stage I patients, as of February 1, 1979, 6 of 28 patients in the combined immunotherapy and immunochemotherapy group died as did 11 of 24 in the total combined group (Stewart TH: Personal communication). The reported difference between the survival curves is highly significant ($P=0.0011$). Serious problems with this study include lack of good evidence of comparability of the groups by histological type of disease and other characteristics and the addition of a nonrandomized control group. Also, no distinction was made between the activities of complete Freund's adjuvant and the tumor antigens which were given together. A confirmatory study is now being conducted by Dr. Takita and co-workers in a trans-Canada trial.

In this trial being conducted by Takita et al., patients with stages I and II disease have been randomized to no further treatment after surgery, complete Freund's adjuvant immunotherapy alone, or complete Freund's adjuvant mixed with tumor antigen as prepared by Hollinshead (Takita H: Personal communication). They have 21 patients in the control arm, 17 in the complete vaccine arm, and 20 in the Freund's adjuvant arm. As of January 1979, only 32% of the controls were still in remission with the longest follow-up of 26 months, whereas approximately 90% of the patients in both therapy arms were still in remission with a longest follow-up of about 32 months. This difference was highly significant. However, no difference was seen between the 2 therapy arms. Thus the effects of immunotherapy with Hollinshead antigen mixed with Freund's adjuvant may relate completely to the effect of Freund's adjuvant and may not be related to the added TAA. Further study of this question will be most important for definitive determination of this point. The trans-Canada trial will not answer this question, however, because there is no Freund's adjuvant alone arm.

Levamisole

Several important studies of immunorestorative therapy have been done on patients with lung cancer treated surgically and with levamisole and thymosin fraction 5. These patients manifest T-cell deficiency by low T-cell numbers, low PHA response, impaired delayed hypersensitivity, etc., and show a relationship between immunocompetence and prognosis. Immune incompetent patients have a poor prognosis relative to competent patients. The immunological data indicate that this area of immunorestoration should receive high priority for therapeutic trials.

A study of levamisole immunotherapy in resectable non-oat cell bronchogenic carcinoma was conducted by a cooperative European group headed by Amery; the original report was made in 1975 (*16*) and the definitive final report in 1978 (*17*). Two hundred eleven patients with resectable lung cancer were treated in a double-blind clinical trial and were randomized to 150 mg levamisole/day in 3-day courses repeated every 2 weeks, compared with a placebo control. The patients were not staged by the current standard but all had resectable disease. Their clinical variables were balanced, including sex, age, skin test results, type of resection, histology, extent of disease, etc. No significant differences between the number of relapses or the number of carcinomatous deaths were seen among the groups of patients. However, if the number of relapses or deaths in the subset of patients which was considered "adequately dosed" (the group under 70 kg in body wt) were evaluated, they were significantly improved in the levamisole group. Thus there were 25 versus 50% relapses and 15 versus 44% deaths. These differences were highly significant. Metastases were particularly delayed in the patients with larger primary tumors. The relapses most affected by levamisole were the hematogenous rather than the regional metastases. This study requires confirmation in a prospective randomized trial so that the drug effect with uniform surface area corrected dose can be evaluated.

A related but not identical trial combining BCG and levamisole being conducted by the Seattle group appears to show no effect at this time (*2*). In addition, Anthony and co-workers (*18*) have entered 318 patients into a randomized trial comparing levamisole to placebo according to the plan of the Amery study, except that the levamisole dose was adjusted for weight. Three centers entered patients into this study, and 135 resectable cases were reported to have been entered between 1975 and 1976. After a minimum of 2 years of follow-up, the diminished survival was highly significant among the 62 patients given levamisole compared with the 73 controls. This survival was entirely related to early nontumor deaths from pulmonary and cardiovascular disease. It is assumed, but not proved, that levamisole therapy was causally related to the noncancer deaths. When these cases were eliminated, there were 46 levamisole and 68 placebo cases which showed no difference in survival. These data directly contradict those of the Amery study. Therefore, decisions regarding further investigations of levamisole in this stage of lung cancer must await definitive analysis of current ongoing evaluations of this agent.

Thymosin

Cohen et al. (*19*) conducted a study of immunorestoration therapy with thymosin fraction 5 in human lung cancer at the NCI and the Veterans Administration Medical Center in Washington, D.C. Patients with oat cell carcinoma of the lung received combination chemotherapy and were randomized into 3 groups of less than 20 patients each; they received no immunotherapy, or 20 or 60 mg thymosin fraction 5/m^2 twice a week. The fraction 5 was given during the 6 weeks of remission induction chemo-

therapy. The 3 groups did not differ in remission rate; the group receiving the high dose of thymosin had a significantly prolonged remission duration and survival compared with the other 2 groups (median survival: chemotherapy plus 60 mg thymosin/m^2 = 450 days vs. chemotherapy and placebo = 240 days). Thus the other 2 groups had virtually no survivors at 500 days compared with about 30% survivors in the 60-mg/m^2 group. Prolongation of survival by immunotherapy correlated with initial low levels of 2 HS glycoprotein and T-cells. The limitations of the above described study are real: They include a few patients randomized to 3 arms of the study and the fact that the patients in the control and low-dose thymosin arms had a shorter survival than has been observed in other studies in which similar chemotherapy alone was used. However, the randomized nature of this study should take this problem into account.

Summary

Immunotherapy of human lung cancer shows promise for active nonspecific therapy with microbial adjuvants and for immunorestoration with thymosin and levamisole. The suggestive trials described above should be considered for eventual attempts at confirmation. Phase I–II studies with careful immunological monitoring are needed. In addition, when definitive phase III studies are done, they must include attention to all of the defects in study design outlined above.

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Breast Cancer

Introduction

Many immunological aspects of breast cancer have been studied, including antibody- and lymphocyte-mediated responses, lymphoid infiltration, and nodal status, and are reviewed in the volume edited by Stoll (*1*). A review is available on "the immunology and immunotherapy of human breast cancer," wherein the rationale for immune modulation in breast cancer treatment was collected (*2*).

The material below is limited to a description of the clinical therapy experiences and randomized trials which support the further development of therapy with agents that modify either specific or nonspecific host immune functions in patients with breast cancer. Results are available only from trials in *advanced* breast cancer, but some trials in potentially "better-responsive" patients are in progress. Controlled clinical trials have been done with 2 immunotherapeutic agents. Trials with the immunostimulating *C. parvum* and with the immunorestorative imidazole levamisole will be reviewed. Although BCG was also used in breast cancer trials, these were not randomized and are included only when they support randomized trials, e.g., with levamisole. Intralesional BCG has been used for

skin implants, but results of controlled trials are not available.

Corynebacterium parvum

The first trial, showing a survival advantage for advanced breast cancer patients given chemotherapy with weekly sc injections of *C. parvum,* was presented by Israël and Edelstein (*3*) in March 1973. Numbers of patients were significant (70 in each arm), but the assurance of comparability in the groups is difficult. This study claimed that the percentage of patients surviving in the immunotherapy group was approximately twice that in controls at 6, 12, 18, and 24 months, and that one-third of the immunotherapy patients survived to 36 months (*3*). Pinsky and colleagues at Memorial Sloan-Kettering Cancer Center (*4*) did a prospectively randomized trial using different chemotherapy. Patients were given CAMF with or without *C. parvum* at the dose and schedule used by Israël, but the *C. parvum* preparation was different. Initial results showed an improved response rate and a survival advantage for CAMF plus *C. parvum* responder patients. In the composite follow-up survival curves for responder patients (fig. 2-1), the cross-hatched area at 6–11 months represents the initial optimistic evaluation of this trial. The fact that the effect which appeared promising was actually relatively transient exemplifies the danger of early and premature evaluation and conclusions.

A third randomized trial to evaluate the addition of *C. parvum* to chemotherapy in advanced breast cancer was done by Mayr et al. (*5*). The initial 76 patients showed some improved response rate (53 vs. 34%); survival at 10 months in the combination was statistically improved (80 vs. 40%). In neither the Pinsky nor Mayr trials was the biological response advantage maintained on prolonged follow-up. Their efforts to conduct chemoimmunotherapy trials with iv *C. parvum* has been difficult because of poor tolerance to the agent by patients.

Levamisole

The second agent investigated in breast cancer is the orally administered antihelminthic, levamisole. Evaluation studies have been done almost exclusively in patients with advanced or disseminated disease. However, Rojas and colleagues (*6*) from Argentina treated primary inoperable breast cancer (stage III) with levamisole following "curative" radiation. This study was initiated in 1972 by Rojas,

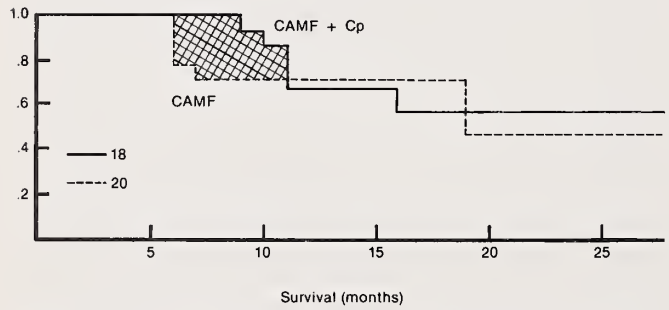

FIGURE 2-1.—Composite follow-up survival curves for responding patients. Cp = *C. parvum.*

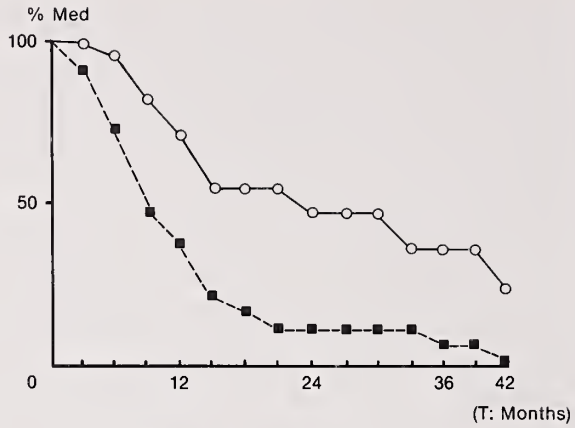

FIGURE 2-2.—Disease-free interval for levamisole and control groups, 20 and 23 patients, respectively. ○ = levamisole, ■ = control.

with a follow-up report by Rojas et al. (*7*). Their patients were alternately assigned to receive placebo or levamisole after radiotherapy. In a separate study, they compared a larger historical control group and additional patients given levamisole. In both the alternate assignment and the historical comparison groups, disease-free and survival intervals were significantly longer when levamisole was administered, as shown in figure 2-2.

An interesting and potentially serious biological difference in the levamisole-treated group concerns the sites of metastases. Metastases to various sites were the same for the levamisole versus the alternate group and for the levamisole versus historical group, with the exception of metastases to the lung (61.5 vs. 21.7% and 47.4 vs. 18.5%, respectively). Repeat trials are being done so that the effect of levamisole on recurrences in stage III following radiation (Olivari) and in stage II primary or recurrent given CAMF (Memorial Sloan-Kettering) can be assessed. Site-specific recurrence information is not available from their ongoing trials. Interim evaluations show that the second of these trials may not confirm the advantage for levamisole claimed in the earlier ones.

Of the 3 studies in which use of levamisole in patients with advanced breast cancer was examined, those of Kleftrom in Finland and Stephens from New Zealand are not published, but data are available from the sponsoring company. The experiences of 231 patients from 3 historically controlled trials at M. D. Anderson Hospital and Tumor Institute have been published (*8*). Levamisole was believed to be well tolerated in all studies; gastrointestinal side effects required dosage reduction in a small percentage of patients. Levamisole-associated agranulocytosis was observed only in the Finland study; 5 of 49 patients developed agranulocytosis which was reversible. The relationship to chemotherapy was not clear. An increased response rate (partial) was reported in only 1 of the 3 studies (Stephens: 15 of 23 for levamisole vs. 6 of 23 for controls). Response of skin metastases was apparently poor. The investigators at the M. D. Anderson Hospital did not improve their FAC response rate by levamisole or by levamisole and BCG. Response duration and survival

were reported to be increased in the Stephens' study but the "responder" group included "stabilized" patients. The sequential nonrandomized studies at M. D. Anderson Hospital showed an improvement in median survival, i.e., from 17 to 28.6 months after the addition of the antihelminthic.

Cell Products

Tumor responses in breast cancer patients treated with cell products have also been observed in preliminary studies. Oettgen (*9*) et al. described partial regression with a 6-month response duration in 1 of 5 patients given multiple doses of dialyzable TF. Gutterman et al. (*10*) reported objective responses of varying degrees in 7 of 17 breast cancer patients given HuLeIF. Unfortunately, IF has not been available for more extensive phase II studies or for randomized trials by the systemic route, nor have comparisons of types of IF been made. The possibilities for treatment by induction of a patient's endogenous IF by other immunotherapeutic agents is just now being considered.

Summary

Initial clinical trials have suggested modest response duration and survival advantages in breast cancer patients given nonspecific bacterial immunotherapy or levamisole in addition to standard therapy. One major cooperative group is now evaluating immunotherapy in stage II disease. It seems appropriate that we encourage the application of new immunological and other host modification therapies in breast cancer, especially if patient response predictors can be ascertained.

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Colon Cancer

Introduction

Compared with other categories of human malignant disease, gastrointestinal cancer has not been investigated intensively for the effects of immunotherapy. Studies of immunotherapy of gastrointestinal cancer can best be considered as 1) therapy to prevent recurrence of Dukes' classes 2B and C disease after surgery and 2) therapy to augment the activity of chemotherapy or other conventional modalities in Dukes' class D disease. In a number of reports of immunotherapy in this cancer, either no data on controls are reported or the total number of patients in a randomized trial was too small, so that the results are uninterpretable. These studies will not be reviewed here.

Adjuvant Trials

In Dukes' class C colon cancer, after surgical extirpation of disease, variable results were obtained with adjuvant chemotherapy (some studies show an increase in survival while others do not). In one of the early immunotherapy studies in this area, Mavligit and co-workers (*1*) randomized 88 patients with class C colon cancer after surgery to receive 6×10^8 BCG organisms weekly for 3 months and then every other week or the same plus monthly courses of 5-FU. They used 73 historical controls for comparison. With a 31-month follow-up (*2*), the disease-free median survival was 35, 34, and 21 months, and the 75-percentile overall survival was 36, 28, 16.6 months, respectively. The 2 therapy arms were not different but both were highly significantly better than the historical control.

This work has been criticized because of the nature of its historical control and the different sources of patients in the treatment versus the control arms. In addition, it has been criticized because the control arm has had a worse survival than the surgery only arm as observed in other centers.

Two trials of MER immunotherapy in Dukes' classes 2B and C colon cancer were designed to prevent recurrence after surgery. Robinson et al. (*3*) randomized 53 patients with these stages of disease to chemotherapy and local irradiation after surgery or the same plus MER immunotherapy. The latter group had a significantly longer disease-free interval and survival than did the chemotherapy group; follow-up period was about 30 months. The immunotherapy was given between courses of chemotherapy. The small numbers of patients and short follow-up preclude definitive evaluation of this data.

More importantly, data from researchers working in other laboratories in this area are potentially conflicting.

Thus Lokich (*4*) entered 394 patients with Dukes' class C colon cancer on a randomized study of 4 arms with surgery alone, surgery plus 5-FU and Me-CCNU, the same plus intradermal MER, and MER alone. At the last report, the median follow-up was 12 months, and the 4 arms did not differ. Longer follow-up will be required for determination of the results, if any, of this regimen.

Immunorestoration immunotherapy has also been attempted in Dukes' class C colon cancer (*5*). Miwa and co-workers treated patients they classified as stage II through III with curative or noncurative surgery with 150 mg levamisole/m^2 per day for 3 days and repeated every 2 weeks. In the more advanced patients, such as stage III, with curative surgery, levamisole increased the 1-year survival from 46 to 73% and in nonresectable disease from 8 to 40%. However, the precise nature of the controls was not clear from the report.

Grandval et al. (*6*) conducted similar studies with levamisole in Dukes' class C colon cancer with equivalent results. This group points out that, whereas a significant effect is observed, the survival curves ultimately come together, and, therefore, it can be assumed the effect is not long lasting.

Trials in Disseminated Disease

In Dukes' class D colon cancer only a few interpretable studies are available. Engstrom and co-workers (*7*) randomized 47 patients with recurrent or local primary advanced colorectal carcinoma to 5-FU or 5-FU plus BCG. The increase in remission rate was not significant (from 21 to 35% partial remission) with the addition of BCG, but the increase in remission duration from 76 to 176 days was statistically significant. This observation requires confirmation in larger numbers of patients.

Both Miwa and associates (*5*) and Grandval et al. (*6*) reported some activity for levamisole in advanced colon cancer as well as in Dukes' class C disease.

In contrast, both Richards and co-workers (*8*) and Moertel and his associates (*9*) found no activity when MER was added intradermally to chemotherapy for Dukes' class D disease, nor did Gough et al. (*10*) for *C. parvum* added to chemotherapy. Bedekian and co-workers (*11*) observed no activity for levamisole added to chemotherapy with regard to remission rate, duration, and survival.

Summary

Thus only hints of activity for immunotherapy are noted in colon cancer. Indeed, one can say that at the present time, BCG and its derivatives are not active in colon cancer. Emphasis should be placed on investigation of new agents and new approaches and not on expansion of marginally positive older studies.

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Gynecological Cancer

Introduction

A number of phase I or uncontrolled studies have implied promise for the use of BCG, BCG plus tumor cell vaccine, or *C. parvum;* however, few historically or concurrently randomized controlled trials have been conducted. This is surprising because virus and/or carcinogen induction has been postulated in the etiology of gynecological cancer, and TAA have been detected.

Ovarian Cancer

An interesting preliminary study was conducted by Hudson et al. (*1*). Patients received autologous or allogeneic tumor cell vaccine containing 2×10^7 irradiated tumor cells mixed with 2×10^5 viable BCG organisms on a monthly schedule. Four of 10 patients after surgical extirpation of ovarian cancer were alive and free of disease compared with only 4 out of 25 historical controls at 2 years. This study is hard to interpret because of lack of data on the selection of controls or patients for treatment.

Crowther and Hudson (*2*) subsequently reported a study, which was a follow-up on that reported by Hudson

et al., in which 10 patients with ovarian cancer treated with a tumor cell vaccine had 21-month survival compared with 12 months for the historical controls.

Juillard and associates (*3*) conducted an interesting investigation of intralymphatic immunotherapy using 1×10^7 to 1.2×10^8 autologous or allogeneic ovarian and other tumor cells. Irradiated tumor cells were injected into the lymphatics of the lower extremities monthly. One patient with ovarian cancer was treated and showed significant regression of tumor after this type of therapy.

Rao and co-workers (*4*) conducted a preliminary trial in advanced chemotherapy refractory patients who were given FAC plus iv *C. parvum* in escalating doses in a 14-day course. Then the *C. parvum* was given sc between chemotherapy cycles; a 50% response rate suggested a benefit from the addition of immunotherapy. However, Wanebo et al. (*5*) subsequently conducted a randomized trial of the FAC regimen versus the same with *C. parvum* as described above but as a pretherapy course. Approximately 20 patients were in each group; response rate, response duration, and survival were identical in the 2 groups.

Of 3 most interesting and provocative recent randomized trials, Alberts (*6*) initially reported in 1977 that the addition of BCG immunotherapy administered by scarification improved the remission rate and duration among patients receiving adriamycin and Cytoxan for disseminated ovarian carcinoma. The BCG was given three times to the upper and lower extremities by rotation between cycles of chemotherapy. Five of 22 controls responded versus 10 of 15 in the BCG-treated group. Recently, this study has been updated, and it appears that BCG significantly improves the response rate, response duration, and survival in ovarian carcinoma (*7*). Fifty-seven of the 118 patients received adriamycin and Cytoxan plus BCG, and 61 received drugs only. Complete and partial remission rates for the combined treatment are 53 versus 36% for the chemotherapy alone ($P=0.05$). After 36 months, survival with the chemoimmunotherapy regimen was 61%, and on the adriamycin and Cytoxan it was 46% ($P=0.004$). The median survival was 23.5 months for the combined therapy and 13.1 for the drugs alone. The prognostic variables were not in favor of the chemoimmunotherapy. Chemotherapy dose and myelosuppression were the same in the 2 groups.

Creasman and associates (*8*) reported that the addition of iv *C. parvum* to melphalan chemotherapy increases the response rate, response duration, and survival of patients with previously untreated metastatic ovarian cancer. The *C. parvum* was given in 4-mg doses iv on day 7 of each chemotherapy cycle. Responses were 53% in the chemoimmunotherapy group, which consisted of 45 patients, and 29% in 63 historical controls. The median survival duration was 24 months in the chemoimmunotherapy group versus 12 months in the chemotherapy controls. This suggestive result requires documentation in additional and properly controlled studies. A randomized cooperative group study is underway.

Cervical Cancer

Patients receiving radiotherapy for stage IV cervical carcinoma have an improved response duration and survival when they receive levamisole immunotherapy concurrent with and subsequent to the radiotherapy compared with a randomized group receiving placebos (*9*).

Majimi and co-workers (*10*) administered BCG CWS to patients with stage IV cervical carcinoma. Although details of the treatment and other therapies are not available, the survival of 28 patients was 12 versus 4 months for 41 who were probably historical controls but were not randomized.

Summary

The above outlined data suggest that confirmatory clinical trials in ovarian cancer are indicated to establish the activity of immunotherapy in gynecological neoplasms. Emphasis in the confirmatory trials should be placed on the entry of an adequate number of patients and on the use of BCG. The results in ovarian cancer suggest that this disease is a good candidate for further studies of BRM.

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Bladder Cancer

Clinical Results

Of the few trials of immunopotentiators in patients with bladder cancer that have been reported, Smith et al. (*1*) conducted a double-blind, randomized study in patients with transitional cell carcinoma. Sixty patients with stage A noninvasive or invasive tumors were randomized blindly to receive levamisole or placebo 2.5 mg/kg daily for 2 consecutive days each week, in addition to standard therapy. The medication was started prior to surgery or radiation therapy and continued for 1–2 years. Monocyte chemotaxis was increased in patients receiving levamisole; however, no therapeutic effect was observed (Smith RB: Personal communication).

Two groups used BCG (Institut de Microbiologie Armand Frappier, Montreal, Quebec, Canada) in the treatment of patients with recurrent superficial bladder cancer. Morales et al. (*2*) treated 16 patients who had at least 2 recurrences in the 12-month period preceding the study and who were treated by resection or fulguration. Within 10 days after cystoscopy and documentation of recurrence, treatment with BCG was instituted. Lyophilized BCG, 120 mg, was suspended in 50 ml of normal saline and instilled into the bladder through a catheter. The patients were instructed to retain the BCG suspension for at least 2 hours. At the same time, 5 mg BCG were administered intradermally by Heaf gun to alternate thighs. The procedure was repeated once weekly for 6 consecutive weeks. Four to 6 weeks after completion of therapy a cystoscopy was performed. Areas suggestive of recurrence were biopsied. When no suspicious areas were seen, multiple random biopsies were done. Follow-up endoscopic examinations were repeated at 3-month intervals. The results (tabulated at 7–30 mo; median, 12 mo) suggest that the treatment reduces the incidence of recurrence (tables 2-1 and 2-2), with only minor side effects.

TABLE 2-1.—*Effect of BCG on bladder tumor recurrence*

Patient No.	Pre-BCG		Post-BCG	
	No. of tumors	Follow-up, mo[a]	No. of tumors	Follow-up, mo
1	2	12	0	30
2	5	12	0	24
3	5	9	0	17
4	7	12	1	16
5	6	12	0[b]	16
6	6	12	0	16
7	3	9	0	12
8	CIS[c]	12	CIS	12
9	CIS	9	CIS	9
10	3	9	0	20
11	SqC[d]	12	SqC	9
12	4	12	0	9
13	2	9	0	9
14	3	9	0	9
15	2	6	0	7
16	2	6	1	7

[a] In patients with long histories of tumor recurrences, only the 12-mo period preceding BCG treatment is recorded; in remaining patients the time between tumor detection and entry into study is indicated.
[b] Recurrence was found in the left ureter.
[c] CIS (carcinoma in situ) was found in several biopsies.
[d] SqC = squamous cell carcinoma.

TABLE 2-2.—*Comparison of recurrence rate within identical observation periods*

Patient No.	Months of observation	No. of tumors	
		Pre-BCG	Post-BCG
1	12	2	0
2	12	5	0
3	9	5	0
4	12	7	1
5	12	6	0[a]
6	12	6	0
7	9	3	0
10	9	3	0
12	9	2	0
13	9	2	0
14	9	3	0
15	6	2	0
16	6	1	1

[a] Recurrence was found in the left ureter.

Douville et al. (*3*) treated 6 patients with recurrent papillomas of the bladder who had at least 2 recurrences in less than 2 years after resection. The patients received 5 mg BCG weekly for 6 weeks first by abdominal scarification, and then 120 mg in 50 ml of saline by intravesical instillation that was retained for 2 hours. A control cystoscopy was done 4–6 weeks after treatment. Four patients showed complete tumor regression, 2 did not (table 2-3). All responders showed local bladder irritation and systemic toxic effects, which were treated with antituberculous drugs in 2 patients. The nonresponders showed no toxic manifestations.

Patients with recurrent stage O, A, B1, and grades I and III bladder tumors received 25 g poly I:C/kg iv every 2 weeks for 1 year in a randomized trial conducted by Kemeny et al. [(*4*); Personal communication]. Thirty-two patients were randomized, 18 received poly I:C. Both groups were matched for age, sex, amount and type of tumor, and duration of disease. After surgical removal of all tumors at the beginning of the trial, 5 of 18 poly I:C-treated patients and 0 of 14 untreated were free of recurrence ($P = 0.04$) at 3 months. However, the difference

TABLE 2-3.—*BCG treatment for recurrent bladder papillomata*

Patient No.	No. of recurrent tumors	No. of tumors after treatment	Complications
1	13	11[a]	None
2	5	0	Major
3	4	4	None
4	10	0	Major
5	20	0	Minor
6	2	0	Minor

[a] Number is not significant.

TABLE 2-4.—*Results of poly I:C in bladder tumors*[a]

Treatment group	Months from starting protocol				
	0	3	6	9	12
Poly I:C	18	5/18	0/17	1/6	3/9
Control	14	0/14	1/14	1/13	1/10

[a] Numerator = No. of patients without tumor; demoninator = No. of patients cystoscoped.

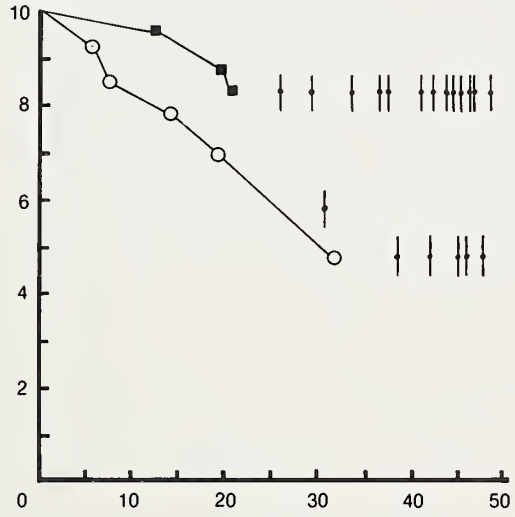

FIGURE 2-3.—Survival from entry of all patients (Wilcoxon test). ■ = patients who received poly I:C (15 of 18 patients alive); ○ = controls (8 of 14 patients alive); ϕ = last follow-up.

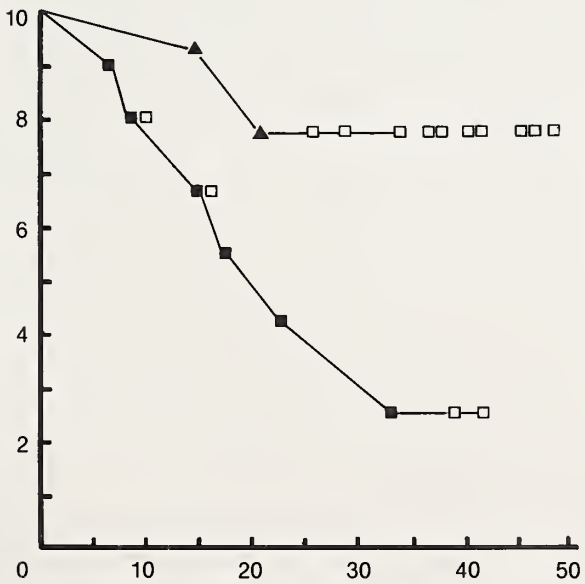

FIGURE 2-4.—Survival from entry of patients with epidermoid carcinoma and/or papilloma (Wilcoxon test). ▲ = patients who received poly I:C (10 of 13 alive); ■ = controls (4 of 10 alive); □ = last follow-up.

became insignificant thereafter (table 2-4). Median survival from the start of treatment was more than 37 months for the treated and 33 months for the control group ($P = 0.003$) as shown in figure 2-3. Figure 2-4 illustrates that when patients with papillomas are excluded, the survivals are over 37 months and 20 months, respectively ($P = 0.002$). Median survival time from diagnosis for all patients ($P = 0.002$) was 135 versus 54 months when patients with papillomas are excluded. Patients whose disease progressed beyond grade II and/or stage B underwent cystectomy. No difference was seen between the 2 groups in the tissue from study entry to cystectomy.

Summary

The results of 2 trials suggest that BCG, administered by both scarification and intravesical instillation, delays recurrence of resected superficial bladder cancer and causes regression of papillomas. Although the reported results are preliminary, they deserve eventual confirmation and extension. The results of this trial suggest an effect of poly I:C on the survival of patients with superficial bladder cancer. The numbers of patients are small, however, and the results warrant retesting.

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Renal Adenocarcinoma

Introduction

Systemic chemotherapy is of marginal value in the treatment of metastatic renal adenocarcinoma; the beneficial effects attributed to chemotherapy are difficult for one to distinguish from the variations often encountered in the course of this disease (*1–3*). Although the initial enthusiasm for hormone therapy (*4–6*) has not been substantiated by recent studies (*7*), progestational agents remain the primary treatment for patients with metastatic renal adenocarcinoma. Clinically useful remissions are unusual, however. True spontaneous partial or complete regression of cancer is a well-recognized but rare event (*8*). However, renal adenocarcinoma is one of the tumor types for which spontaneous regression is most frequently reported (*4*). This suggests that the host immune system may modulate the growth of this tumor. Thus a number of

investigators have attempted immunotherapy in patients with metastatic renal adenocarcinoma.

Clinical Results

Laucius et al. (*9*) had a patient with pulmonary metastases from a renal adenocarcinoma who experienced subjective improvement and objective tumor regression with BCG and megestrol acetate therapy. In a subsequent phase II trial, no objective responses were noted among 15 patients treated with 160 mg megestrol acetate/day for 56 days and 5 immunizing doses of BCG intradermally, every 2 weeks for five times. They concluded that this regimen was not therapeutically beneficial.

Ten patients with metastatic renal adenocarcinoma (*10, 11*) were treated with BCG (obtained from the Institut Armand Frappier, Montreal, Quebec, Canada). Following a single intradermal inoculation, a 40-mg dose was administered weekly for 4 weeks, twice monthly for 2 months, and then monthly with a multipuncture (Heaf gun) apparatus. Four of 10 patients were reported to have responded objectively; regression of pulmonary metastases was noted in 3. The X-rays were reproduced, and in at least 1 patient, evidence of regression was clear. It must be pointed out that X-rays reproduce poorly. In the fourth patient, supraclavicular lymph node metastases regressed. Two of 4 patients had, in addition to the signal lesion, other disease which remained stable. Remissions were of short duration (5–8 mo). These data suggest biological activity, but the therapeutic effect of BCG was modest.

Minton et al. (*12*) gave 9 patients with metastatic renal cancer weekly intradermal injections of BCG (source and dose unknown). Four patients showed "clinical control" of their disease, and 1 had a complete remission of 4 years' duration.

Using polymer particles prepared from autologous tumor material, Tykka and co-workers (*13*) treated 21 patients with renal carcinoma metastatic to the lung. Inoculates mixed with tuberculin or *Candida albicans* as adjuvants were injected intradermally at 4-week intervals. Five patients had complete disappearance of lung metastases in 3 to 11 months of treatment. No relapses were encountered during a mean follow-up time of 16 months. X-Rays are reproduced for each patient and are impressive. However, several questions arose. One patient was suffering from toxoplasmosis, but the chest X-ray was not typical for this disease. A second patient was given a short course of progestational agent therapy with the immunotherapy. However, during this period the lung metastases progressed. A third patient had bone metastases which did not respond to immunotherapy.

In an attempt to reproduce the work of Tykka and associates, Neidhart et al. (Neidhart HA, Murphy SG, Hennick LA: Submitted for publication) noted 2 complete and 2 partial remissions among 30 patients.

Schapira et al. (*14*) treated 7 patients with measurable metastatic renal cancer using weekly injections of 10^7 irradiated (10,000 rad) autologous tumor cells admixed with 37.5 g of *C. parvum* and noted 1 complete and 1 partial remission of pulmonary metastases lasting 18 and more than 5 months, respectively.

A report by Horn and Horn (*15*) on 4 patients (all in one family) with renal carcinoma showed that, after the pulmonary metastasis in 1 patient was treated with plasma transfusions from an uncle who was free of disease after surgery, the pulmonary lesion regressed completely. As described and depicted, this solitary lesion was "ill-defined."

Summary

Twenty-one of 84 (25% patients with metastatic renal adenocarcinoma) treated with immunotherapy are reported to have responded. With the use of stringent criteria of complete remission of a clearly defined lesion or well-documented partial remission, at least 9 of these 84 patients (10.7%) responded favorably; this exceeds the spontaneous regression rate. Remissions were limited to pulmonary metastases and were short for some patients. However, the results seem superior to those achievable with chemotherapy. Equally impressive is the lack of negative reports, although they are less likely to be published.

Research in this area should be encouraged. Large-scale phase II trials of BCG seem warranted. In addition, renal adenocarcinoma should be high priority for phase I and II immunotherapy trials in man.

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Head and Neck Cancer

Introduction

The history of immunotherapy for head and neck cancers exemplifies the principles, pitfalls, and prospects for progress in this difficult therapeutic area. This disease meets the 3 major criteria for immunotherapeutic manipulation as reviewed by Browder and Chretien (*1*). Specifically: 1) Head and neck cancer patients demonstrate decreased immunologic competence, i.e., reduced skin test reactivity, low numbers of circulating T-lymphocytes, poor responses to mitogens, etc. The fact that standard therapies further reduce immune competence may lead to a poor prognosis due to loss of host immunologic reactivity. 2) The epidermoid tumors are presumably immunogenic and are also associated with the development of serologic responses to DNA viruses. 3) These tumors are accessible for tissue sampling and measurement and are often of limited size when discovered; thus they conform to the minimal residual disease criterion.

Clinical Results

Control with surgery and radiation therapy in patients with advanced disease has been difficult; response rates have been modest with MTX alone or with combinations such as BACON. In one nonrandomized study by Donaldson (*2*) a favorable response rate (62%) and long survival were observed in a group of patients given chemotherapy plus nonspecific immunotherapy with BCG. This clinical result in a pilot study was sufficiently exciting to stimulate investigators to do randomized studies to confirm the beneficial effects. Confirmatory trials in advanced, recurrent, or metastatic disease were not entirely encouraging (*3*). Researchers then took additional steps to identify other immunotherapeutic agents, conduct trials in groups with better prognoses, identify subsets of patients who may benefit from host-response modification, and to model combined modality therapy to take advantage of positive clues from the historical experience. These trials and development steps are summarized below.

Three prospective randomized trials conducted to confirm the immunotherapy benefit first reported by Donaldson (*2*), enrolled 131 patients with advanced, recurrent, or metastatic disease. All patients were assigned to receive intradermal BCG or BCG by scarification and received chemotherapy weekly or biweekly. Chemotherapy was either MTX or the 5-drug combination regimen (BACON). Objective tumor responses were not different for the chemotherapy alone versus combination therapy.

The Yale/West Haven Veterans Administration study reported by Papec et al. (*4*) revealed that only 7 of 35 patients had partial responses; the Mayo Clinic trial (*5*) resulted in only a 10% response rate. Responses were better in patients with oral cavity primaries, and a good pretrial performance status contributed the expected effect, but an advantage for BCG was not demonstrated with the small numbers of patients studied. The M. D. Anderson trial of BACON plus BCG reported by Richman and co-workers (*6*) also shows a site-dependent response rate: 5 of 6 for nasopharynx versus 3 of 11 for oral, oropharynx, and tonsil. This study does show a significantly longer survival for patients given BCG, as shown in figure 2-5 (30.5 vs. 13.5 wk median; $P=0.014$).

Minimal benefit with dermal BCG in patients with advanced head and neck cancer and reports of positive trials with levamisole in lung and breast cancer encouraged new trials with this host-immunomodulating imidazole in head and neck cancer. Two significant trials have been reported in which 150 mg levamisole was given daily for 3 days every other week. This therapy was associated with minimal toxicity, except for a low incidence of neutropenia. A study by Wanebo et al. (*7*) enrolled 65 patients with neoplasms at all sites in all stages. Initial evaluation revealed statistically significant results only for the small subset of patients with oral primaries and for stage II disease. Although longer follow-up decreased the detectable benefit for oral primaries, the small numbers of patients used and the subset analysis suggest that definitive analysis of the larger numbers now entered into this study be awaited before definitive conclusions are drawn. Although earlier investigators enrolled patients with neoplasms at all sites and stages, generally advanced, the study by Mussche and colleagues (*8*) at the University of Ghent is a refinement. In this randomized study, they added the levamisole dose and schedule indicated above to *curative* surgery and radiation for epidermoid carcinoma of the larynx and hypopharynx and also used a placebo. The recurrence rate for the 12 placebo and 12 treated patients is shown in figure 2-6. The difference in survival for these 24 patients had not reached statistical significance at the time of reporting.

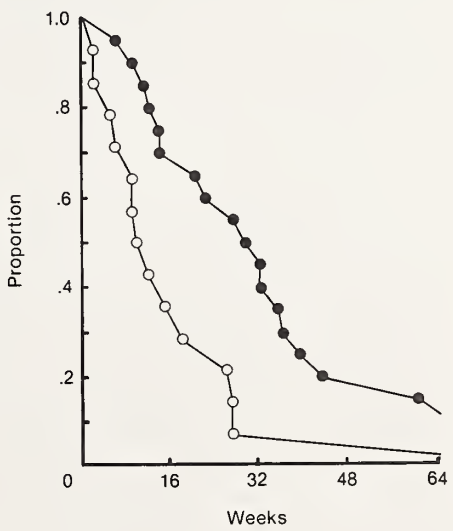

FIGURE 2-5.—Survival of patients: BACON alone (○), no survivors vs. BACON and BCG (●) 2 of 20 survived.

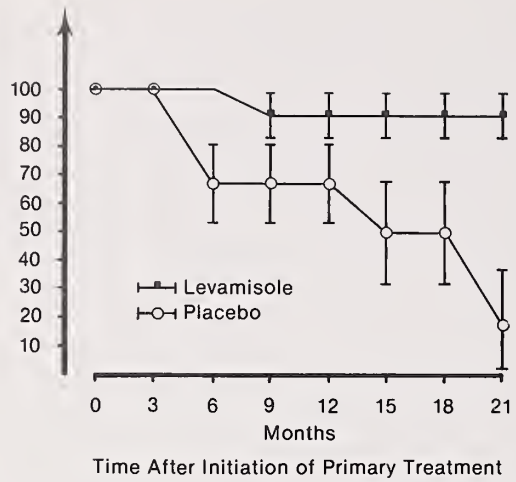

FIGURE 2-6.—Recurrence rate for 12 patients treated with placebos and 12 with levamisole.

Nonspecific immunotherapy is also being given in the surgery with and without *C. parvum* trial by Schuller and colleagues at the Medical College of Virginia (*9*). Preoperatively, 7 mg *C. parvum* is administered intralesionally, 3.5 mg monthly for 12 months, and then every 2 months for 6 times it is given sc postoperatively. Only *surgically curable patients* are enrolled in Schuller's study; currently, 108 patients are randomized by stage. Unfortunately, no additional data are available on this large patient population.

A third combination immunotherapy protocol (with radiation), by Suit and colleagues at the Massachusetts General Hospital (*10*), based the route, dosing schedule, and immunotherapeutic agent on detailed modeling using an immunogenic murine fibrosarcoma. Patients with biopsy proven squamous carcinoma were randomized to receive conventional treatment alone or in combination with *C. parvum* (initial paratumoral injection, then iv). This study has been completed. To date, neither tumor response, time to recurrence, nor survival has been increased by the addition of *C. parvum* to radiation treatment of any stage of head and neck cancer. As these data have not been published, additional details cannot be disclosed. However, this appears to be a definitive negative study.

Summary

Although BCG and *C. parvum* have not been demonstrated to be of therapeutic benefit to patients with head and neck cancer, some positive effect has been observed by the addition of levamisole to conventional treatment for early stage cancers of the oral cavity, larynx, and hypopharynx. Furthermore, studies on the addition of immunotherapy to other potentially curative regimens are justified. These results also justify the continuing research of newer immunotherapies or other BRM in patients with head and neck cancer, particularly in the adjuvant setting.

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Malignant Melanoma

Introduction

Malignant melanoma has a natural history which suggests that immunological intervention by the host significantly alters its timetable of progression and metastasis. It is well-known that decades may separate initial appearance of a primary lesion and its subsequent widespread dissemination. During the radial phase of growth, with lymphocytic infiltration prominent, a melanoma may persist indolently for years, to be followed by sudden vertical growth activity, diminution of infiltrating lymphocytes, and rapidly developing metastases (*1*). Furthermore, cases of spontaneous regression of primary melanomas as well as metastatic disease are well documented (*2*). Some of these regressions have followed influenza infection, rabies vaccination, and blood transfusion and have been attributed to an attendant immunological response. For these and other reasons, malignant melanoma has interested immunobiologists and immunotherapists. Patients with cutaneous malignant melanoma have been subjects of intense efforts toward therapeutic modulation of the host's immune response.

Local Immunotherapy

The animal studies of Zbar and associates (*3*) stimulated interest in the use of intralesional BCG in the treatment of cancer. Working with an inbred strain of guinea pigs and a transplantable hepatocarcinoma, these investigators demonstrated that intralesional BCG can induce regression of

established dermal tumor transplants as well as stimulate tumor-specific transplantation immunity. They found that tumor regression requires an intact immune system and direct contact between BCG and tumor cells. Subsequent studies in this system indicated that intralesional BCG treatment of dermal tumor transplants can control nodal and visceral metastases in selected situations (*4*).

In 1970, Morton et al. (*5*) determined that the injection of live BCG organisms directly into cutaneous melanoma deposits could induce tumor regression in immunocompetent patients. Following his example, intralesional BCG therapy for metastatic malignant melanoma was adopted by a large number of investigators. The pooled data of 14 investigators (table 2-5) reveal that, in approximately 66% of the patients treated by intralesional BCG, injected nodules regressed, and, in 21% of patients, regression of uninjected nodules (these were mainly in close proximity to injected lesions) was also observed. Twenty-seven percent of the patients, who experienced complete remission of all clinically evident disease, were almost exclusively immunocompetent individuals with small tumor burdens limited to the dermis.

The results with the use of intralesional BCG in the treatment of metastatic melanoma are both encouraging and disappointing. That this modality can cause regression of injected and uninjected lesions has been documented by many investigators. These observations are the most clearly demonstrable and readily reproducible evidence for an antitumor effect of immunotherapy. On close scrutiny, one sees that many questions remain unanswered. Individual experiences are small, treatment techniques are poorly described, and response data are incomplete. Some remissions can be long (up to 8 yr), but what is the median? What are the clinical characteristics of those patients who respond? What are the best strains of BCG, the best dose and schedule? Although hundreds of patients are being treated with this modality, no results have been published recently. This topic was not included in the 1976 International Conference held at the NCI. To encourage investigators to define further the response parameters would be worthwhile; then, if warranted, this treatment should be made uniformly available to the appropriate patient population.

TABLE 2-5.—*Immunotherapy of melanoma with intralesional BCG*[a]

Source of BCG	Regression of nodules[b]		CR[c]	References
	Injected	Uninjected		
Glaxo, Tice	25/29[d]	6/36	11/36	(*5–8*)
?	1/4	1/4	1/4	(*9*)
Tice	7/9	2/9	2/9	(*10*)
Glaxo	0/1	0/1	0/1	(*11*)
Tice, Glaxo	15/25	2/25	2/25	(*12*)
Tice, Glaxo	12/15	11/14	7/15	(*13–15*)
Tice	3/7	1/7?	?	(*16*)
?	2/8?	?	?	(*17*)
Tice	1/2	0/2	?	(*18*)
Connaught	2/3	1/3	?	(*19, 20*)
Tice	4/6	2/6?	3/6	(*21*)
Pasteur	7/11?	0/11	?	(*22*)
Glaxo	1/2	0/1	0/1	(*23*)
Glaxo, Tice	5/5	?	?	(*24*)
	85/127(66%)	26/120(21%)	26/98(27%)	

[a] Data are from the BRM Subcommittee Interim Report of September 1979.

[b] Values are No. of patients showing regression of nodules/total No. of patients treated.

[c] CR = Complete response.

[d] Regression on a per patient basis was observed in only 29 patients.

TABLE 2-6.—*Local immunotherapy of melanoma with miscellaneous nonspecific immunostimulants*

NSI[a]	Regression of nodules[b]		CR[c]	References
	Injected	Uninjected		
PPD	1/1	1/1	?	(*27*)
PPD, DNCB	5/9	0/9	?	(*28*)
DNCB	1/1	?	1/1	(*29*)
DNCB	164/255[d]	?	?	(*24*)
Vaccinia	9/10	0/10	6/10	(*30*)
Vaccinia	4/30?	?	3/30	(*31*)
Vaccinia	1/1	0/1	0/1	(*32*)
Vaccinia	1/1		1/1	(*33*)
Vaccinia	1/1	?	1/1	(*34*)
MER	12/18	?	6/18	(*35*)
C. parvum	6/14[e]	0/14	3/14	(*36*)
C. parvum	31/86[f]	?	?	(*37*)
Nitrogen mustard	6/10[f]	?	?	(*24*)
CWS+P3[g]	11/23[e]	0/23	?	(*37*)
CWS+P3	6/15	4/15	?	(*38*)

[a] NSI = nonspecific immunostimulants.

[b] Values are No. of patients showing regression of nodules/total No. of patients treated.

[c] CR = complete response.

[d] Value represents No. of nodules showing regression in 11 patients.

[e] Several patients had tumors other than melanoma.

[f] Value represents No. of nodules showing regression in 5 patients.

[g] P3 = trehalose dimycolate.

Thomas (*25*) described a patient with melanoma metastatic to the bowel that regressed following oral BCG therapy continued for 4 months. In this latter instance, close approximation of BCG organisms with the visceral lesion resulting in regression is likely. Silverstein et al. (*26*) have noted regression of an injected bladder melanoma metastasis. As methods of introducing BCG into visceral lesions are developed, we may define a role for intralesional BCG in the treatment of metastases beyond the skin.

Results of the use of immunostimulants other than BCG topically and intralesionally in the treatment of dermal lesions of recurrent malignant melanoma are summarized in table 2-6. The agents in these small series of patients have some efficacy, and response rates appear similar to those obtained with intralesional BCG. Other agents, e.g., PPD, MER, *C. parvum,* and DNCB are likely to have mechanisms of action similar to BCG. An obvious advantage over BCG is that these are nonviable materials. Direct comparisons against BCG have not been attempted except for the study of Cohen et al. (*24*) using DNCB. As that trial contained only 11 patients, few definitive con-

clusions were possible. Once the efficacy of BCG has been detailed, it seems reasonable for one to undertake direct comparisons with possibly superior materials.

The mechanism of action of vaccinia, on the other hand, may be different because Milton and Brown (*31*) observed that presensitization of patients to vaccinia virus appears to lessen its efficacy in achieving nodule regression. It has been suggested, and there is some confirmatory evidence, that vaccinia superinfects tumor cells preferentially, inducing cytopathic changes (*32*). The immunized host might eliminate the viruses before significant infection of tumor cells could be achieved.

Systemic Immunotherapy

Micrometastatic disease.—Because immunotherapy appears to be most effective in experimental animals with small tumor burdens, investigators have initiated studies to determine whether immunostimulants might prolong the disease-free interval in melanoma patients who have undergone definitive surgery but are at high risk of recurrence, i.e., with occult, micrometastatic tumor deposits.

Results of several historically controlled trials have suggested that postoperative adjuvant BCG was effective in prolonging remission duration and/or survival. These conclusions have been questioned because of the difficulty we encounter in demonstrating the comparability of current treatment and historical control groups. Moreover, we cannot compensate for changes in conventional treatment over time. Pinsky et al. (*39*) conducted a randomized prospective comparison of BCG (Tice; $4–6\times10^7$ viable U; tine technique; weekly for 52 wk, every other wk for 26 wk, and monthly for 1 yr) versus no treatment following surgery in stage II melanoma (table 2-7). No benefit was detected. The patient population was sufficiently small that a clinical benefit may have been overlooked. Furthermore, the dose of BCG was substantially less than that for which benefit was claimed in earlier historically controlled trials. Finally, BCG was not given in the lymphatic drainage of the primary tumor.

Table 2-7 also provides data on a randomized prospective trial in stage II melanoma (*40*) in which no treatment following surgery and BCG (Tice, $1–2\times10^8$ by tine weekly for 12 wk, then biweekly) were compared. Although BCG has not prolonged remission duration, it has favorably influenced survival following recurrence, which has been attributed to an alteration of recurrence patterns toward less ominous sites in treated patients.

Several other groups are evaluating adjuvant immunotherapy and/or chemotherapy in melanoma. The International Group for the Clinical Study of Melanoma (*42*) is conducting a 4-arm study in stage I–II melanoma: No treatment versus BCG (lyophylized Pasteur, 75 mg percutaneously) versus DTIC versus DTIC plus BCG. The results at the time of an interim report (March 1978) are shown in figure 2-7. All 3 treatment arms were performing better than the untreated control. Wood et al. (*43*) evaluated DTIC, BCG, and DTIC and BCG in stages I and II melanoma patients following potentially curative surgery. Recurrences were as follows: DTIC, 6 of 20 (30%); BCG, 5 of 28 (17.9%); DTIC plus BCG, 0 of 22. Survival of patients treated with DTIC and BCG was superior to that of patients treated with either agent alone. An untreated control was not included. The study has now been reduced to 2 arms. Twelve percent of the patients on the BCG arm have had recurrences compared with 3 of 41 (7.3%) patients treated with DTIC plus BCG. These data suggest a therapeutic advantage for DTIC and BCG. This has not been confirmed by the European trial. More detailed reports of these 2 trials will be required before this difference can be explained. Stimulation of additional research in this area does not seem warranted.

Several investigators have attempted preoperative intralesional adjuvant immunotherapy. Everall and associates (*44*) injected the primary melanoma with vaccinia virus in 23 patients 2 weeks before definitive surgical therapy. The disease-free intervals for these patients were compared (actuarial method) with those achieved by a group of 25 patients who received standard surgical therapy. Allocation to treatment and control groups was not random. The two groups were comparable in age but the vaccinia virus-treated group contained significantly more tumors of the superficial spreading type. Tumors were graded I-V with respect to degree of dermal infiltration. Grades I–III (low risk) and IV–V (high risk) were compared; no statistical differences between these distributions were observed.

TABLE 2-7.—*Nonspecific immunostimulation in the maintenance of remission in melanoma patients in stages I, II, III*

Parameter	Treatment group					
	BCG			Prospective randomized control		
	Investigator (reference)			Investigator (reference)		
	(*39*)[a]	(*40*)[a]	(*41*)[b]	(*39*)	(*40*)	(*41*)
No. of patients	24	45	51[c]	23	46	41
Median remission duration	69 wk	14.4 mo		76 wk[d]	10.1 mo[d]	
Median survival	125 wk	23.7 mo		125 wk[d]	13.8 mo[e]	
Percent recurrence at 10 mo			22%			20%

[a] Treatment was for stage II.
[b] Treatment was for stages I, II, and III.
[c] Patients received levamisole.
[d] *P* was not significant.
[e] $P=0.01$.

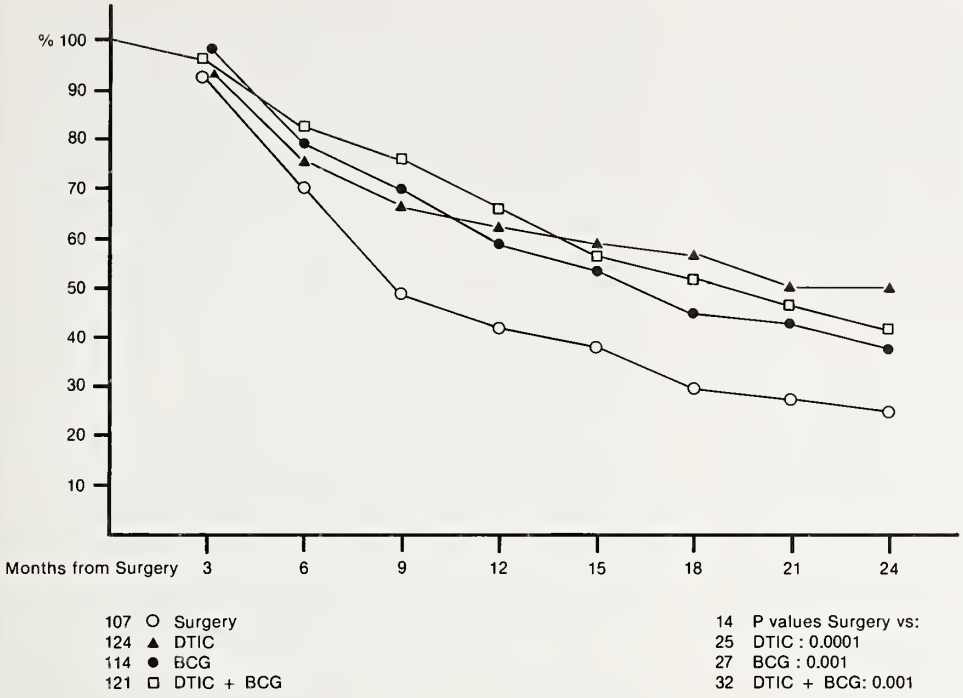

FIGURE 2-7.—Disease-free interval in all evaluated patients.

Relapse rates are shown in table 2-8. The data suggest that vaccinia virus-treated patients may have benefited therapeutically.

Of the 37 clinical stage I patients Castermans-Elias et al. (*45*) treated with DNCB, 23 presented with their primary melanoma intact (clinical diagnosis). They applied 2 mg DNCB to the tumor for 48 hours (repeated twice at weekly intervals). Eight to 10 days later, a standard surgical excision was performed. A second group of 14 patients presented after excisional biopsy of the primary. Here the DNCB was applied to the scar of the incision. The results, after 3 years of observation and as presented in table 2-8, show that the statistically significant difference in relapse rates suggests an advantage for preoperative DNCB. However, diagnosis in the treated group was on clinical grounds, and microstaging was done after several applications of DNCB. A properly designed trial of preoperative intralesional adjuvant immunotherapy is needed.

Macrometastatic disease with immunotherapy alone.—Laucius and co-workers (*46*) treated 18 patients with surgically incurable metastatic malignant melanoma with a mixture of irradiated (15,000 rad) autologous tumor cells ($1–2\times10^8$) and BCG (Glaxo, $2–4\times10^6$, 5×10^6 organisms), which was injected intradermally (in 5 divided doses) every 2 weeks for five times. Four of 18 (22%) patients who could be evaluated achieved objective remissions. It was concluded that this treatment regimen does not have general clinical application because the remissions were infrequent,

TABLE 2-8.—*Intralesional preoperative adjuvant immunotherapy*

Patient status	Period, yr	Treatment					
		Excision + vaccinia[a]	Excision only[a]	*P*	DNCB[b]		
					Before surgery	After surgery	*P*
No. of patients		25	23		23	14	
Relapses, %							
All patients	1	5	20	NS			
	2	5	30	NS			
	3	15	35	NS			
Stages IV,	2	5	50	0.05			
V only	4	20	50	0.05			
Relapses, No.					0/23	4/14	0.05
Deaths					0/23	0/14	NS

[a] *See* (*44*).
[b] *See* (*45*).

of short duration (median, 3 mo), and occurred only in patients with minimal, nonvisceral tumor burdens. Nonetheless, biological activity was demonstrated.

Using the Hollinshead tumor antigen (500 to 1,000 g polyacrylamide gel, region A) on an unspecified schedule, Arlen et al. (*47*) treated 15 patients with advanced melanoma. Six of the 15 achieved tumor-free status. One pulmonary metastasis regressed completely, and 3 patients showed regression of modal disease. One with extensive pelvic tumor had regression of visceral seeding that allowed later resection of a localized necrotic tumor mass. Although this brief report is encouraging, it is not clear if simultaneous chemotherapy was used.

Nineteen patients with stage III melanoma were treated with iv BCG by Orefice et al. (*48*). A single dose of lyophilized Pasteur BCG ranging from 2×10^7 to 3×10^8 viable U was given in 500 ml saline infused over 5–6 hours. Three of 16 patients showed objective regression of more than 50% of the original volume lasting 2–5 months. The objective remissions were induced at the higher (2×10^8) BCG doses, although toxicity was severe. Only skin, subcutaneous, and lung metastases responded. Partial remissions were also observed by Israël et al. (*49*) in 2 of 4 patients with advanced melanoma treated with iv *C. parvum* (4 mg/day, 5 days/wk).

Falk and co-workers (*50*) noted objective tumor regression in 2 of 7 patients with disseminated melanoma, treated repeatedly with oral BCG. One of these 2 responders had bone metastases.

Of 2 groups who used viral oncolysates in the treatment of disseminated melanoma, Wallack and associates (*51*) treated 3 melanoma patients with a virus-lysed tumor cell vaccine, and 1 of 3 responded favorably. This patient had hepatomegaly and abnormal liver function tests; however, no evidence of liver metastases was seen after biopsy. The patient received 6 oncolysate injections at 2-week intervals and experienced normalization of liver size and liver function studies. Murray et al. (*52*) administered melanoma cell oncolysate prepared with Newcastle disease virus to 13 patients. Although 6 were reported to have had regression of skin or lymph node disease, review of the patients' case reports revealed no clear evidence of a definite antitumor effect.

Six patients with advanced melanoma were treated with plasmapheresis; 1 patient with subcutaneous and lung metastases showed partial regression (*53*) but died 8 months later. The subcutaneous lesions measured up to 5 cm in diameter.

Overall, the results achieved with systemic immunotherapy alone are modest. The most encouraging antitumor effects were achieved with iv BCG or *C. parvum*. That any response to biological response modification was seen was impressive and should encourage further research on this modality in this stage of disease.

Macrometastatic disease with chemoimmunotherapy.— The results of relevant chemoimmunotherapy trials in melanoma patients with disseminated diseases are summarized in table 2-9. Gutterman and co-workers (*54*) evaluated the efficacy of DTIC plus Pasteur BCG by scarification in 89 patients, using as controls a retrospective group of 111 patients treated with the drug alone. Patients receiving DTIC–BCG exhibited a response rate significantly greater than patients treated with DTIC alone ($P=0.05$). Mean survival was also greater in the chemoimmunotherapy group ($P=0.001$). Patients with lymph node metastases and no evidence of visceral disease were particularly benefited by the DTIC–BCG regimen, demonstrating a remission rate of 55% compared with 18% for controls ($P=0.025$). An augmented response in metastatic nodules regional to BCG scarification sites was also noted. The authors speculated that drainage of BCG into regional lymphatics and local tissue potentiated chemotherapy at those sites.

Randomized, prospective studies have also yielded evidence for an enhanced efficacy of chemoimmunotherapy regimens compared with chemotherapy alone in melanoma patients. Costanzi et al. (*55*) determined the contribution of BCG to a triple drug regimen (BHD) of BCNU, hydroxyurea, and DTIC. Addition of BCG to the 3-drug regimen did not result in enhanced overall response

TABLE 2-9.—*Chemoimmunotherapy in disseminated melanoma (stage III)*

Investigator (reference)	Treatment	No. of patients	Response rate, %[a]	Median duration response	Median duration survival	Remission rate/1 yr, %
(*54*)	DTIG–BCG	89	27	6 mo	7 mo	20
	DTIC, historical controls	111	14.4	5 mo	5 mo	0
	P		0.05		0.001	
(*55*)[b]	DTIC–BCG	119	19			
	BCNU, hydroxyurea, DTIC–BCG	150	29			
	BCNU, hydroxyurea, DTIC	82	35			
	P		NS			
(*56*)[b]	DTIC–CPP–*Corynebacterium parvum*	55	29	13 wk		
	DTIC–CPP	65	18	15.6 wk		
	P		NS	NS		

[a] Response rate = complete and partial remissions.

[b] This was a randomized study.

rates. However, BCG patients over age 60 and patients with predominantly pulmonary or lymph node metastases had significantly higher response rates. Analysis by age, sex, and site of disease indicated that patients with pulmonary metastases had significantly longer remission ($P=0.04$) and survival ($P=0.05$) with BCG. Moreover, BCG seemed to yield longer survivals in patients over 60 years of age ($P=0.01$). These observations need confirmation in trials specifically designed to evaluate these points, particularly because only the above described subsets of patients were shown to benefit from immunotherapy.

Presant and co-workers (*56*) observed no difference in response or survival between treatment groups when they evaluated *C. parvum* added to a drug regimen of DTIC and CPP. In a regimen which included BCG and allogeneic tumor cells, Mastrangelo et al. (*57*) failed to demonstrate a greater efficacy of the chemoimmunotherapy regimen.

The addition of currently available immunotherapies to chemotherapy regimens may yield a small biological effect, which is significant in some subgroups and of sufficient magnitude to be of continued clinical research interest particularly with newer and more active agents.

Summary

The regression of injected and, occasionally, uninjected dermal melanoma metastases is the most clearly demonstrable and readily reproducible evidence for an antitumor effect of immunotherapy. Because of the rarity of patients with dermal metastases (without visceral metastases) individual studies are small. Variations in treatment regimens between and among studies have prevented a conclusion regarding the best therapeutic regimen. As this is an effective form of treatment, the NCI should encourage a large-scale study so that response and treatment parameters can be defined more exactly. The Food and Drug Administration could then be petitioned to accept this as a standard treatment for this select group of patients.

The role of microbial products as postsurgical adjuvants is slowly being resolved. It appears that a slight therapeutic effect is discernible. Properly designed adjuvant studies of newer treatments should be encouraged. Based on the animal model and fragmentary preliminary clinical data, presurgical immunotherapy appears to be a promising area of research.

An overall assessment of the results of chemoimmunotherapy use in patients with disseminated disease suggests a modest biological effect as evidenced by improved survival. This observation should be pursued with newer and potentially more effective BRM. The biological and clinical characteristics of cutaneous malignant melanoma make this disease an ideal model for future studies of BRM.

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Nonmelanocytic Skin Cancer

Interest in tumor immunotherapy was at least partly rekindled by the pioneering work of Klein and co-workers (*1*). They used various nonspecific immunostimulants (e.g., PPD, BCG, DNCB) to treat primary cutaneous neoplasms as well as skin metastases from noncutaneous primaries.

Regression of injected lesions was noted as follows: breast cancer, 7/14; Kaposi's sarcoma, 5/6; and mycosis fungoides, 8/13. Van Scott and co-workers (*2*) used topical dilute nitrogen mustard and DNCB to treat mycosis fungoides. The degree of tumor regression was directly correlated with the magnitude of the DTH response. However, the antitumor effect was demonstrable in desensitized patients with nitrogen mustard, which indicated a direct cytotoxic effect. This remains a fruitful area of continued research.

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Lymphomas

Introduction

Lymphomas represent a reasonable target for clinical immunotherapy trials for the following reasons: a) reactivity to putative TAA has been reported (*1*) in patients with lymphoma; 2) the tumor burden can be significantly reduced by conventional radiation and/or chemotherapy; 3) the immune stimulant used, e.g., BCG, is processed in proximity to the tumor tissue; 4) the effect of any therapy may be easily followed, especially as reflected by changes in the size of lymph nodes; and 5) recent advances in clinical and pathological staging have identified the most important clinical prognostic variables, so that comparable patients can be represented in experimental and control groups and patients most appropriate for study can be identified.

Unfortunately, the latter characteristic also represents a major pitfall for immunotherapy studies. For example, Sokal et al. (*2*) reported that BCG vaccination decreased and delayed relapses in patients with stage IA or IIA lymphoma who were entered on study in 1965 to 1967. The results, reported in 1974, suffer from the marked heterogeneity of histological types of lymphoma (including Hodgkin's and non-Hodgkin's subtypes), the changes in clinical staging procedures that occurred through the years, and the changes which occurred in the conventional therapeutic approaches to the treatment of the various subpopulations of patients. Therefore, the results are not interpretable.

Clinical Results

Bacillus Calmette-Guêrin.—In a recent report, Hoerni and co-workers (*3*) suggested that BCG may be effective in lymphoma. Patients with non-Hodgkin's lymphoma with aggressive histology ("central blastic, immunoblastic, lymphoblastic, unclassifiable . . . and centroblastocentrocytic diffuse-type") or with advanced (stage III or IV) disease and/or "first or repeated relapsed" were treated to complete remission by combination chemotherapy and radiotherapy and were then randomized to receive no further treatment or to receive BCG. The chemotherapy consisted of COP or COP plus adriamycin or VM26; BCG was given by skin scarification on a 5×5-cm area through 20 fresh dermal scarifications, each 5 cm long. The scarifications were performed weekly for 3 years or until relapse.

Overall, relapses occurred in 13 of 23 controls and 3 of 20 patients given BCG ($0.01 > P > 0.025$). The only significant difference is seen in stage I patients with relapse in 0 of 9 BCG-treated patients versus 5 of 9 controls. Comparable figures for stage II are 2 of 5 versus 4 of 6, respectively, and for stage II and IV, 1 of 6 versus 4 of 8. Eight of 23 patients died in the control group, whereas only 1 died in the treated group (highly significant).

Although the results are encouraging, serious problems hamper evaluation of the results: 1) Data on histologic staging are not adequate. 2) Clinical staging was not optimal because no routine marrow examination was performed, and patients staged clinically as I or II may, in fact, be IV. 3) The chemotherapy regimens varied between the 2 groups. 4) During the chemoradiation therapy, patients in both the BCG-treated and control groups "were submitted to several BCG vaccinations before randomization." 5) Eight of 20 BCG-treated patients were female, whereas only 3 of 23 in the control group were female. 6) Seven patients in each group had already relapsed one or more times and thus the patient population was heterogeneous.

It is unfortunate that the study for stage I and II disease has been stopped. The results remain encouraging but involve too few confirmed patients and should therefore be repeated with additional and more homogeneous patients.

The best study and the most promising results have been reported on the use of BCG as adjuvant therapy in non-Hodgkin's lymphoma by Jones et al. (*4*). Patients with stages III and IV disease without prior chemotherapy and histologically classified according to the Rappaport criteria were randomly assigned to 3 remission induction regimens (stratified for nodular or diffuse histologies): 1) COP plus bleomycin; 2) CHOP plus bleomycin; and 3) CHOP plus BCG. High-viability Pasteur BCG was reconstituted from lyophilized vaccine ($6 \pm 4 \times 10^8$ viable U) just prior to use and administered by scarification on days 8 and 15 of each 21-day treatment cycle. After 8 courses of treatment, patients with complete remissions confirmed by thorough restaging were rerandomized to receive either no further treatment or BCG by scarification at monthly intervals for 18 months.

Although BCG was well tolerated, 52% had local inflammatory reactions. Mild toxicity recurred in 36% of the patients and required BCG dose reduction, 11% exhibited moderate or severe toxicity with local or regional abscess formation, and 1 patient developed suspected BCG disease.

As of December 1978, 447 patients had been totally evaluated and the results are as follows: 1) Complete remission rate was higher with nodular than with diffuse lymphomas. 2) Chemoimmunotherapy produced a higher rate compared with chemotherapy, but the differences were not significant. (For diffuse disease the complete remission rate was 48% with CHOP + bleomycin and 54% with CHOP + BCG; for nodular lymphomas, the figures were 67% and 75%, respectively.)

As of December 1978, 334 patients have been registered on the maintenance arm in complete remission and 303 can be evaluated. The following results were reported: 1) Duration of complete remission was longer for nodular than for diffuse lymphoma. 2) Maintenance BCG after remission had no effect on the rate of its duration. 3) For diffuse lymphoma, the duration of complete remission was longer for the 2 CHOP regimens than for COP plus bleomycin, but the difference between CHOP and BCG versus CHOP and bleomycin was not significant. 4) For nodular lymphoma, the duration of complete remission was virtually identical for all 3 remission induction arms with a median of about 2 years and a rate of relapse constant at about 20–25% per year. 5) The overall survival of patients with the nodular type was significantly superior to that of those with diffuse lymphomas. 6) The survival of patients with diffuse lymphomas to whom the CHOP regimens were administered was superior to that of those given COP plus bleomycin. 7) Although the survival of patients with diffuse lymphoma was not significantly different with CHOP plus BCG than with CHOP and bleomycin, the survival curves diverged after the first year, an observation which must be followed closely (fig. 2-8).

Finally, the most critical observation is that survival of patients with nodular lymphoma who received CHOP plus BCG was better than that of patients treated with CHOP and bleomycin ($P=0.08$) and superior to that achieved with COP and bleomycin ($P=0.002$). *See* figure 2-9.

Thus this large study with what is probably the most homogeneous population of patients from the standpoint of histological and clinical staging and uniformity of treatment shows that the 1) complete remission rate is not

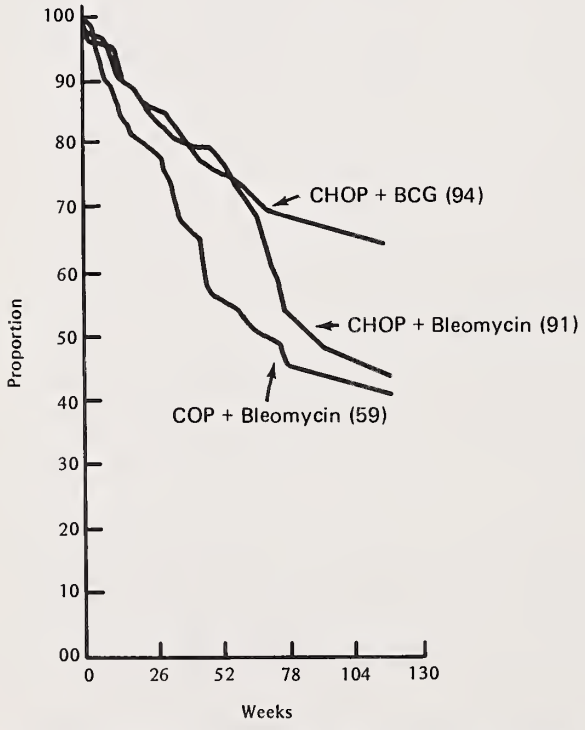

FIGURE 2-8.—Survival by treatment, diffuse lymphoma.

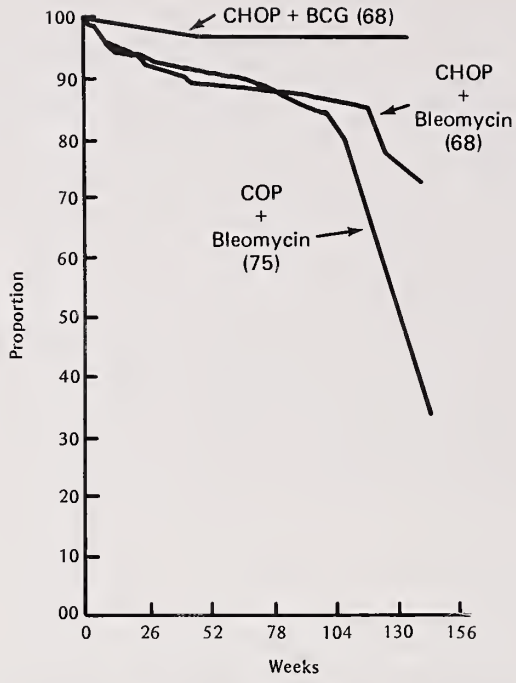

FIGURE 2-9.—Survival by treatment, nodular lymphoma.

significantly higher for chemoimmunotherapy, and 2) duration of complete remission is not prolonged by BCG administered during or after it has been achieved but that survival of patients with nodular lymphoma is apparently significantly better ($P=0.08$) when CHOP is given with BCG instead of bleomycin. (Some improvement in survival in diffuse lymphoma is also possible.) That the improved survival of patients with nodular lymphoma with BCG was noted only when the BCG was given with the initial chemotherapy but not when the BCG was administered only after a complete remission had been induced is most interesting.

The results, though not significant at the less than 0.05 level, are encouraging. Although it would have been preferable to have one arm be CHOP alone without bleomycin or BCG, this cannot be used as a significant criticism of the results, unless one postulates that the bleomycin somehow had a deleterious effect on survival. This is not inconceivable, and data to rule that out are probably available.

The apparent improvement in the survival of patients with nodular lymphoma treated with BCG is, according to the authors, consistent with reports in which survival of patients with other diseases, such as AML, was associated with BCG therapy despite the absence of a significant increase in the duration of complete remission. One wonders whether the results in AML might reflect an increased tolerance or acceptance of chemotherapy after relapse and therefore a greater overall antileukemic effect of larger amounts of chemotherapy administered after relapse in patients given BCG. This interpretation could only be applied with difficulty to nodular lymphoma in which no association between complete remission and survival is apparent, and therefore the ability for one to

administer larger amounts of chemotherapy after relapse should presumably have little effect on overall survival. The Southwest Oncology Group has apparently terminated the present study and has embarked on one with the same induction scheme but randomization to CHOP alone, CHOP plus levamisole, and CHOP with levamisole and BCG.

Interferon.—Non-Hodgkin's lymphomas have recently been reported to respond to HuLeIF. Merigan et al. (*5*) recently treated 6 patients with a 30-day course of 10^7 IU of IF/day im. The average blood IF levels achieved were 299 ± 35 U/ml. All patients developed a depression in granulocyte counts which returned to normal when therapy was stopped.

Three patients had diffuse histiocytic lymphoma. None responded to IF therapy. Three others had nodular lymphocytic, poorly differentiated lymphoma with measurable lesions. Two of the patients exhibited dramatic resolution of abnormal retroperitoneal lymph nodes as seen on lymphangiography beginning in the third week of therapy. One of them was noted to have an excess of circulating monoclonal B-lymphocytes before therapy that disappeared after treatment but recurred 3 months after it was stopped; the patient's lymphosarcoma cell leukemia was then treated with chemotherapy. The other patient had lymphoma cells in the marrow before the IF therapy that then disappeared, and the patient had no evidence of progression of disease in the marrow, the retroperitoneal nodes, or anywhere else at 9 months when the results were reported. A third patient exhibited less striking reduction in retroperitoneal node size but showed complete regression of a 5×5-cm subcutaneous mass. She remained well with no evidence of progression of disease and without any further therapy at 6 months at the time their article was published (*5*).

Gutterman and associates (*6*) also treated non-Hodgkin's lymphoma patients with HuLeIF and noted no response in the 1 patient with histiocytic lymphoma. However, of 6 with nodular, poorly differentiated lymphocytic lymphoma treated with IF, 2 had complete remissions, 1 had a partial remission, i.e., greater than 50% reduction in the lymph node size, 2 exhibited a reduction of less than 25%, and 1 progressed.

Summary

Lymphomas represent an appropriate target for therapeutic studies with BRM. A review of the studies to date reveals encouraging results with BCG and preliminary but nevertheless encouraging results with HuLeIF. Confirmation of these effects is clearly indicated.

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Leukemia

Acute Myelocytic Leukemia

Several randomized trials show an advantage in our adding immunotherapy to chemotherapy in adult patients, but, unfortunately, we have no results of immunotherapy given alone as maintenance. The Medical Research Council trials reported in final form by Galton et al. (*1*) show increased survival in the group given Glaxo BCG vaccination and 10 courses of killed (irradiated) allogeneic pooled leukemia cells with chemotherapy over the group given only chemotherapy as maintenance. The frequency and duration of remissions were not improved, but remissions were more easily reinduced in the immunotherapy-treated group. Seventy-one patients in complete remission were randomized between maintenance chemotherapy and chemoimmunotherapy between January 1, 1973, and June 30, 1976, in the largest single randomized trial of immunotherapy in acute leukemia yet reported. Results were exhaustively analyzed by several methods, including comparisons of significance between medians, life-table analysis, and log-rank P values. It was concluded that, although the duration of the first remission was slightly longer in the group given immunotherapy with chemotherapy, it was not statistically significant. The immunotherapy plus chemotherapy patients lived 22 weeks (median) after relapse, whereas those receiving chemotherapy lived only 14 weeks. Log-rank analysis revealed a P of 0.08 (statistically insignificant), but the wide 95% confidence interval was consistent with no effect, and, alternatively, with a *halving* of the death rate among relapsed patients as well. The same was true of the rate of survival after randomization (i.e., from onset of remission) that was perhaps increased by twice or not at all, as concluded from the wide 95% confidence interval. Thus the results of even this careful trial are inconclusive but point toward an advantage in patients given immunotherapy during the maintenance period.

Increased survival (20 vs. 10 mo) was also noted by Reizenstein and co-workers (*2*) in a similarly designed study with Glaxo BCG plus 10^9 viable allogeneic myeloblasts given weekly as immunotherapy and chemotherapy maintenance. Eighty-nine patients were entered in this study, 43 of whom had a remission. Of these 43 patients, 30 were randomized between the 2 treatment arms. The duration of remission was 8 months for patients receiving chemoimmunotherapy versus 5 months for the chemo-

therapy group. Thus, once again, survival was increased out of proportion to the prolongation of the remission. The exclusion from randomization of 13 patients in complete remission and the unusually short duration of remission in the chemotherapy group make it more difficult for one to draw definitive conclusions.

Nonspecific active immunotherapy, with BCG alone (i.e., without tumor cells) has also achieved some success in patients with AML. Vogler and Chan, who are with the Southeast Oncology Group, obtained improved survival when BCG (Tice) administered by multiprong vaccination was used intensively for 4 weeks before beginning maintenance chemotherapy with MTX (*3*). In this trial, a randomized comparison was made with a group given MTX maintenance alone. The median duration of remission was only 13 weeks for patients receiving chemotherapy alone versus 25 weeks for those with BCG pretreatment; both are short but significantly different nonetheless. Although many patients relapsed subsequently and died, the group given BCG had significantly more survivors at 20 months, with a 93-week median survival compared with 78 weeks for the chemotherapy group.

By iv administration, BCG alone improved the duration of remission and of survival when added to chemotherapy during maintenance of remission (*4*). Treatment with Glaxo BCG at approximately $4\text{–}9\times10^5$ organisms led to a 64-week median survival, as opposed to 47 weeks for the group receiving only chemotherapy. Relapses occurred in both groups, but only in the iv BCG group were reinductions possible (6 of 13 or 1 of 13). The real risks of dissemination of BCG, the high fever uniformly produced, and the potential for "septic" hypotension militate against this route, however. Use of a less toxic component of BCG iv may be a more feasible approach in the future.

Preliminary results of studies of 2 types of immunotherapy at Mount Sinai School of Medicine have strongly suggested a beneficial effect. Once again, immunotherapy was added to maintenance chemotherapy. In one, neuraminidase-treated leukemia cells conferred an advantage in duration of remission (*5*), particularly *without* an immunological adjuvant. When 10^{10} allogeneic myeloblasts were injected intradermally at several sites, improved duration of remission was observed in 31 patients (22 mo) versus that in 17 controls (6 mo). Myeloblasts with MER led to 11 months median remission in 22 patients. A concomitant control group from a Leukemia Group B study has a 10-month duration of remission and was included in the graphs for comparison. Despite the author's explanations that the short remission of controls here was due to an unusual method of calculation [*see* (*5*), p 353], the duration *was* shorter than in most studies. Nevertheless, neuraminidase-treated myeloblasts added significantly to remission when given alone and probably did not when given with MER. Duration of survival was not given in these studies but presumably was longer by at least the increased length of the remission, which is characteristic of leukemias. In a second investigation, intradermal immunization with MER may have benefited a subgroup of anergic patients, but the overall effect was negligible (*6*). In fact, the considerable toxicity of MER has led these investigators to abandon its use (Bekesi JG: Personal communication).

An interesting but uncontrolled small study on *Pseudomonas* vaccine, the adjuvant properties of which are probably due to endotoxin, has shown that median survival and the number of individuals remaining alive in complete remission was increased when the vaccine was given to patients in remission (*7*). Six of 13 patients first treated in 1970–72 are free of disease in 1979, compared with 1 of 17 who did not receive the vaccine (Oettgen HF: Personal communication). Studies on *C. parvum,* levamisole, and other agents cannot be judged now because they appeared only in abstract or preliminary form.

As a broad generalization, patients with AML who were given chemoimmunotherapy as maintenance have usually had longer durations of remission, but, in most studies, they have survived longer. Among the factors contributing to this is a greater ease of reinduction by chemotherapy in patients given chemoimmunotherapy than in those maintained on chemotherapy alone. Subtler changes in the host's resistance to infection, to myelosuppression by chemotherapy, etc., as a result of the activity of immunization, particularly with nonspecific adjuvants such as BCG may also play a role in increasing survival.

Because chemotherapy maintenance in AML has not been clearly demonstrated to prolong the duration of survival, a study of immunotherapy alone as maintenance versus no maintenance might now be in order. Such an investigation would clarify the role of immunotherapy per se, without compromising good medical care, if intensive chemotherapy were first given for induction and consolidation.

Childhood Acute Lymphocytic Leukemia

Mathé et al. (*8*) reported that immunotherapy with either Pasteur strain BCG by scarification (8 patients), killed pooled lymphoblasts (5 patients), or a combination of the two (7 patients), improved the survival of a proportion of children with ALL. Of these 20 patients treated with immunotherapy in 1969, 8 are still alive and free of disease 10 years later, 7 of whom are in the initial remission. An expanded group of 100 patients treated since 1969 with immunotherapy, standardized to include both BCG and killed (irradiated) allogeneic lymphoblasts, has had a 50% 5-year survival, and 25% of the total remain in their first remission. Apparently, no chemotherapy was given to this group of 100 patients after the initial induction of remission. A number of groups have attempted without success to confirm Mathé's findings, but none of them reproduced the parameters of the original work. Perhaps for that reason, the role of immunotherapy in the treatment of ALL remains unsettled.

The administration of BCG alone, by scarification, failed to improve survival in the hands of the Children's Cancer Study Group (*9*). Inasmuch as most of Mathé's patients received immunotherapy with leukemia cells, with or without BCG, this does not constitute an adequate refutation of the earlier study. Also a minute amount of BCG was given, less than 1% of Mathé's dose. The Concord group also found no differences among groups

given BCG alone or intermittent MTX alone; no maintenance therapy was administered after intensive initial cytotoxic therapy (*10*). Also, allogeneic tumor cells were not given in this study either.

Poplack et al. (*11*) found no advantage to adding BCG and allogeneic irradiated leukemia cells to maintenance MTX when 56 patients in remission from ALL were randomly assigned to these 2 groups. Rates of relapse and duration of remission were similar to both groups. This study too, with its inclusion of chemotherapy in both arms, is also not a direct reproduction of the study of Mathé et al., and the same criticism of the dominating influence of chemotherapy on results can particularly be levied against it. A recent continuing controlled trial has shown no difference between groups given either immunotherapy or chemotherapy as maintenance. Andrien [for the European Organization for Research on the Treatment of Cancer (*12*)] compared BCG and tumor cells versus MTX and 6-mercaptopurine in 122 randomized patients by a scheme designed to follow Mathé's original schedule. The median duration of remission was the same in both groups. Unfortunately, because of the belief that unmaintained remission by chemotherapy was unethical, even after a year's intensive consolidation, investigators did not withhold therapy from any group to see whether either form of maintenance was better than none. However, assuming that in ALL (as opposed to AML) maintenance therapy is useful in prolonging remission, it appears that immunotherapy was as good as chemotherapy in prolonging remission and thus survival. Median duration of survival of these patients has not been determined, but it is interesting that no death from infection occurred in the BCG plus tumor cell-treated group, whereas 5 deaths occurred in the chemotherapy group.

The excellent durations of survival now uniformly achieved in childhood ALL, with nearly 50% permanent cures, has made the extra margin of improvement afforded by immunotherapy difficult for us to ascertain. If one agrees with the insistence that even after intensive consolidation chemotherapy must be given as maintenance, a trial randomizing between maintenance chemotherapy and chemoimmunotherapy, similar to those in AML, seems reasonable. Ideally, a 3-armed study of chemotherapy, chemotherapy plus immunotherapy, and immunotherapy alone would be most interesting; irradiated allogeneic cells would be given with a nonspecific adjuvant as the immunotherapeutic regimen.

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ADDENDUM: UPDATE OF CLINICAL STUDIES: 1979–81

During the last 2 years, approximately 100 fairly definitive studies of human cancer immunotherapy have been published. These reports fall into three broad categories, including phase I–II studies, therapy administered as adjuvant treatment after surgical extirpation of disease, and therapy administered as adjuvant treatment between courses of chemotherapy for disseminated disease. Most published reports still deal with intact microbial adjuvants such as BCG, crude microbial extracts such as CWS preparations, and studies with the immunomodulating agent levamisole. Of increasing prominence are those of individual cases or small series of patients being treated with one of the partially purified IF preparations. In addition, important reports are beginning to appear on the clinical application of monoclonal antibodies in cancer therapy.

Most of the studies conducted during the last 2 years predominately involve therapy of patients in 3 disease

categories, i.e., lung cancer, breast cancer, and malignant melanoma. Few deal with acute or chronic leukemia, or cancers of the colon, bladder, head and neck, and ovaries. In addition, approximately 15 published reports that can be evaluated are in the area of phase I and II studies.

In solid tumors, data of most published works involve the systemic administration of BCG, MER, or *C. parvum,* and also levamisole. The problems with these approaches, recognized in the initial report of the BRM Committee persist. Thus, in apparently well-controlled, randomized clinical trials in which similar tumor therapy regimens and doses and schedules of immunoadjuvants are used in patients with the same diagnosis, both efficacy and lack of efficacy have been demonstrated. Of 31 studies that have been appraised, 18 appeared to demonstrate that the above-noted modalities of immunotherapy improved remission rates, remission durations, or survival compared with the appropriate controls. The overall conclusion is that these relatively weak modalities of immunotherapy do not have a reproducible activity.

Further reports on AML and ALL have appeared that indicated immunotherapy with BCG alone or combined with allogeneic irradiated tumor cells did indeed prolong remission and survival or increased the rate of subsequent second remission induction (*1, 2*). In addition, investigators from 2 laboratories have produced evidence that tumor burden reduction, if not complete remission, has been induced with leukocyte or lymphoblastoid IF in patients with leukemia (*3, 4*).

The studies of intravesical BCG in patients with bladder carcinoma continue to indicate potent therapeutic activity. In 4 studies involving a total of 147 patients, decreased recurrences have been unequivocally demonstrated, as have conversion of urine cytology from positive to negative and actual tumor regression (*5–8*). In the latter instance, complete remissions ranged from 20 to 40%, and partial remissions occurred in an additional 10–20%. Intravesical BCG is well tolerated; the results confirm the preliminary observation that this approach to immunotherapy is effective.

During 1979–81, therapeutic trial results with IF have accumulated slowly. Reports of trials are still insufficient in regard to numbers of patients for an evaluation of the degree of efficacy, nor are the numbers of reported phase I studies which would indicate an optimal dose and schedule of treatment significant. In the available studies, biological assays of serum IF are being used for pharmacology. Immunochemical or molecular serum IF assays are not available, nor are those for the detection of IF receptors on tumor cells. Continued limited supply problems, in addition to the above factors, prevented a definitive analysis of therapy with this group of agents. Partially purified leukocyte IF (*3*) or partially purified lymphoblastoid IF (*4*) has been administered to a limited number of patients with leukemia, including those with diagnoses of ALL, AML, CML in blast crisis, and CLL (*9*). Most patients receiving higher doses of IF have shown measurable tumor burden reduction. Patients with AML have been placed in a phase I trial of partially purified lymphoblastoid IF and have been escalated to doses of over 20 million IU/day by continuous infusion (*4*). Tolerance and toxicity have been described and have been clinically acceptable. Tumor burden reduction was noted without evidence for complete remission. In the American Cancer Society-sponsored studies, approximately 20% of the treated patients have shown partial remission in both breast cancer (*10*) and multiple myeloma (*11*) that confirm earlier reports. A small series of 9 patients with malignant myeloma were treated and 1 showed a partial remission (*12*). Nineteen patients with non-oat cell carcinoma of the lung were treated, with no responses noted after 1 month of therapy (*13*). A series of studies of patients with multiple myeloma by the Swedish group reported use of different preparations of partially purified leukocyte IF. Large variations in response rates from study to study were noted (*14*). Anecdotal cases of neuroblastoma and nasopharyngeal carcinoma and other tumors have been shown to respond to these IF (*15*). Whereas studies with the recombinant DNA-produced IF have been initiated, we know of no definitive results that have been published. The studies with IF have determined that various routes are safe and usually tolerable, including im, iv, and intrathecal.

A limited number of investigations have also been done with IF inducers. One of the most exciting is the use of poly A:U by Lacour and co-workers (*16*) as adjuvant therapy to prevent recurrences of stages I–III breast cancer. The patients did not receive chemotherapy, and poly A:U was administered at random. The increase in the disease-free interval was highly significant, and survival was comparable to that produced by adjuvant chemotherapy. Several phase I reports of the use of poly ICLC have been published (*17, 18*) in which toxicity and a lack of therapeutic efficacy were noted. A phase I trial of the pyran copolymer derivative MVE-2 indicating tolerability and some immunostimulating activity has recently been presented (*19*).

Several promising agents in the phase I area are worthy of mention. Work continues with the intracavitary injection of microbial agents. Two reports (*20, 21*) describe a high proportion of partial and complete remissions with *C. parvum* mainly in patients with pleural effusions related to lung or breast cancer. Generally, these responses were only seen in reduction or disappearance of pleural fluid with no effect on other manifestations of disease. In a few instances, peritoneal effusions have also responded. Early studies with BCG CWS intralesionally have shown that it has an antitumor activity similar to that of BCG (*22*). A phase I trial indicated its tolerance and tolerability. More recently, Japanese workers have described *Nocardia* CWS, and phase I trials demonstrated that, although it has local toxicity, it generally is as well or better tolerated than intralesional BCG CWS (*23*). Phase I studies with IF inducers are ongoing.

One of the most promising areas of phase I work at this time is in the field of adoptive immunotherapy. Immune RNA has been used in the adjuvant setting in renal carcinoma with possible prolongation of the disease-free interval after surgery (*24*). More clearly, immune RNA treatment by the method of in vitro incubation of patients' lymphocytes with guinea pig immune RNA to renal carcinoma, followed by reinfusion of the lymphocytes, has resulted in complete and/or partial remissions in 3 of 7

patients so treated (*25*). Another approach to adoptive immunotherapy is for one to grow PBL in large cell numbers using TCGF. Phase I studies with the iv infusion of such cells resulted in characterization of their physiology and pharmacology (*26*). They appear to localize in the lung(s) for approximately 4 hours after which they redistribute to liver and spleen. However, that study was not oriented toward evaluation of therapeutic efficacy.

A number of studies of active specific immunotherapy in renal carcinoma have also appeared. The authors continue to suggest that, when tumor antigens are administered coupled to PPD or in a lipoidal adjuvant, tumor regression in patients with metastatic renal carcinoma can result (*27, 28*).

The potential for chemotherapy has recently been greatly augmented by the development of monoclonal antibodies. Patients with melanoma have been treated with monoclonal antibody to melanoma cell surface-associated antigen (*29*), and lymphocyte leukemia–lymphoma patients have been treated with monoclonal antibody to T-cell surface antigen (*30*). Notable results of these studies are 1) a lack of toxicity, 2) lack of development of anti-mouse immunoglobulin antibody or immune complexes, and 3) some degree of therapeutic efficacy with tumor burden reduction in the patients with the leukemia–lymphoma. Using conventional antibody, Order et al. (*31*) demonstrated that antitumor therapy with iodine-131 coupled to antiferritin antibody can induce partial remission in patients with hepatoma.

Finally, the approach of plasma immunoabsorption for removal or disruption of circulating immunosuppressive immune complexes has been investigated to a limited extent and appears to be associated with at least temporary regression and hemorrhagic necrosis in patients with breast cancer.

Although the current clinical trial results are not encouraging, the prospects for the development of effective immunotherapy appear to remain high. This optimism is based on the potential of various approaches which offer theoretical or practical promise resulting from animal model work. Synthetic immunoadjuvants, macrophage activators, and IF inducers are being entered into clinical trials. The potential to deliver these to these desired tissues selectively through their encapsulation into liposomes offers us a unique new approach to this modality. Through the development of monoclonal antibodies it now becomes possible for us to focus on the purification of large amounts of relevant tumor cell surface antigens for immunotherapy trials. Encouraging results with immune RNA will be explored in significantly greater detail. Our ability to grow large numbers of cytotoxic T-lymphocytes with antitumor specificity and indeed to clone these cells now becomes a reality because of the availability of TCGF. Work with IF and lymphokines suggests that lymphokine and cytokine therapy with agents, such as lymphotoxin, TNF, CSA, and immune or gamma IF, will all be ongoing shortly. Finally, monoclonal antibodies have potential or antitumor use, by themselves or as carriers for chemotherapy, toxins, or radioisotopes. Whereas only mouse–mouse hybridomas are readily available at this time, there is great promise that human–human hybridomas will be developed during the next few years. These will do away with at least the theoretical problems associated with the use of mouse immunoglobulins in man.

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SECTION III:

The Interferon System

THE INTERFERON SYSTEM[1]

The IF are a family of inducible secretory glycoproteins produced both in vivo and in vitro by eukaryotic cells in response to viral infections or other stimuli (*1*). They appear to be intercellular messages in that, upon binding to specific cell receptors (*2–4*), they can direct cells to alter the expression of some of their specialized functions (*5*), i.e., to express an IF-induced phenotype. Therefore, IF have aptly been called "inducible inducers."

Characteristically, IF induce in cells a broad spectrum resistance to viral infections (*6*). This easily quantifiable property is used by scientists to measure the biological activity[2] of IF preparations.

Production and action by IF involve the workings of several structural and regulatory genes (Ruddle F: Personal communication). These can be activated rapidly, and their products, including IF, are detected within hours of their induction (*7*). Because of the rapidity with which it can be mobilized and its effectiveness, this "interferon system" appears to be an important first line of defense against viral and other infections (*8*). In addition, perhaps owing to their cell growth inhibitory and immunomodulatory effects, IF also appear to confer a degree of protection against aberrant cells, including neoplastic cells.

HISTORY

The existence of IF was discovered in 1957 (*9*). Over the ensuing 22 years, their complex biological effects were studied in in vitro and in vivo systems with the use of progressively more purified and potent preparations (*10, 11*). Nowadays, IF effects can be studied at the molecular level in heterologous or cell free systems (*12–15*), and several animal and human IF have recently been purified to homogeneity (*16–19*). It was demonstrated unequivocally that the effects obtained earlier with impure preparations, with the exception of perhaps only one (Pitha P: Personal communication), can be attributed to IF molecules themselves [(*20, 21*); Gresser I: Personal communication]. Therefore, these natural substances appear today to be not only of considerable scientific interest but also to have remarkable potential as prophylactic and therapeutic agents. Generally, interest is keen among researchers in producing them in quantity to explore their possible clinical use. This process has begun with early encouraging results (Edy VG: Personal communication).

[1] This is the Section submitted with the Interim Report of the Subcommittee on Biological Response Modifiers of the Board of Scientific Advisors of the Division of Cancer Treatment, NCI, which was dated September 30, 1979. Since this date it has been edited only with no updating.

[2] One unit of IF activity is defined as the reciprocal of that dilution of an IF preparation which inhibits virus plaque formation by 50%. When an International Standard Reference preparation is run in parallel in the assay, units can be converted into, and expressed as IU.

INDUCTION AND PRODUCTION OF INTERFERONS

Production of IF by cells requires the interaction of an IF inducer with a cell sensitive to it. This reaction triggers the derepression of genetic information (*22, 23*) that leads to the transcription of the *IF* gene(s), the appearance of IF mRNA, its translation into protein, the latter's posttranslational modification, and the secretion of the IF glycoprotein. During an early period, varying from 1 to 8 hours depending on the inducer-cell system, these processes can be aborted by inhibition of DNA-dependent RNA synthesis. Later, IF biosynthesis is sensitive to inhibition of protein synthesis.

The kinetics of IF induction and production vary with different inducer-cell systems, with IF appearing extracellularly generally within 4–12 hours, maintaining maximum levels for about 12 hours, and then decreasing abruptly to return to undetectable amounts.

Type I IF, such as those virally induced in mouse L-cells, and human fibroblast IF and HuLeIF are produced following this general kinetic pattern. Other types of IF designated as type II and produced by leukocytes exposed, e.g., to mitogens, appear much later: PHA-stimulated B-cells produce a type II IF 5 to 7 days after induction. Such differences between various inducer-cell systems suggest that either different genes can be induced or different mechanisms may be triggered under different circumstances [for review, *see* (*24*)].

Only 1 animal laboratory cell line has been described which is incapable of producing IF (*25*). The capacity to produce IF seems common to all vertebrates and has even been attributed to some plants (*26*), which attests perhaps, to the IF system's positive selective value in evolution.

Unstimulated cells do not secrete IF nor do they contain detectable IF mRNA. Evidence exists from the study of several inducer-cell combinations that IF biosynthesis results from a true inductive process, i.e., that it represents de novo synthesis (*24*).

The production of IF can be significantly enhanced by treatment of certain induced cells with metabolic inhibitors at appropriate times (*27*). This phenomenon, called "superinduction," is believed to be due to the inhibition of the synthesis of a regulatory protein which occurs before maximum IF synthesis. Under superinducing conditions, the production of IF is delayed but reaches higher levels and is prolonged. Superinduction has been exploited to maximize IF production in fibroblast-type cells (*11, 28*). Superinduction of human leukocytes has not been possible, perhaps because the synthesis of the regulatory protein and that of IF are synchronous.

A phenomenon called "hyporesponsiveness" to repeated IF induction has often been described. Following IF production, cells go into a refractory phase, characterized by failure to respond to a new induction for a period which, for mouse cells, lasts for 28–48 hours and possibly several days for human cells. Hyporesponsiveness, which apparently occurs following stimulation by all inducers and is one of the obstacles to their effective clinical use, is not understood nor has it been circumvented, except, perhaps, in mice, through the use of co-administration of prostaglandin (Stringfellow D: Personal communication).

THE INTERFERONS

The definition of what constitutes an IF includes first of all that it be capable of inducing an antiviral state in cells undergoing RNA and protein synthesis.

Native IF molecules are glycoproteins; however, it has been demonstrated that part of or all their carbohydrate moiety is not necessary to biological activity (*18, 29*). The active part of these molecules appears to be a polypeptide(s).

Rather than being species specific, as has long been believed, IF usually exert more activity on the cells of their species of origin but they also have a definite, characteristic host range or cross-species activity and are often capable of exerting their biological activities on cells of phylogenetically distant species (*25*).

In their species of origin, IF are neither antigenic nor allergenic. Neutralizing activity in sera of patients inoculated for many months with $1–3\times10^6$ IU of HuLeIF/week has not been detected (*30*). This property is important in the clinical use of IF because it makes possible treatment of patients repeatedly at high-dose regimens, without eliciting neutralizing antibody or allergic reactions (*31, 32*).

However, IF are antigenic in heterologous species, even in those in which they are active biologically (*33–37*). Antisera with high titer neutralizing antibody (10^6) have been obtained against mouse IF (*38*) and HuLeIF (*35*). Such antisera are capable of inactivating both the antiviral and nonantiviral activities of IF which suggests that these share a common antigenic site or are located on closely related sites.

Several molecular species of IF can be produced by the same animal species and even by the same kind of cell (*39*).

In man, HuLeIF and human lymphoblastoid IF are indistinguishable antigenically (*40*), although each type contains a small and different amount of fibroblast-type IF (1 and 10%, respectively) of the total activity (*39, 41, 42*). Antiserum to human fibroblast IF has no activity against the leukocyte type (*42*).

Also distinguishable on the basis of their resistance to low pH, HuLeIF and lymphoblastoid and fibroblast IF are resistant to pH 2 and are designated as Type I IF. Those produced by mitogen- or antigen-stimulated lymphoid cells are labile at pH 2 and are designated as Type II, which are also antigenically distinct from Type I (*43, 44*). Intermediate types have also been described [(*24*); Epstein L: Personal communication]; the present terminology was recently changed.

When mouse L-cell IF is analyzed by SDS–PAGE, its activity is distributed in 2 peaks corresponding to mol wt of 22,000 and 38,000 (*45–47*). This is true for mouse IF induced in various cells either in vitro or in vivo. The human fibroblast IF migrates in SDS–PAGE as 1 band at 20,000 daltons (*17, 42, 48*), whereas HuLeIF activity is resolved under the same conditions into 2 components corresponding to 15,000 and 21,000 daltons (*5, 17, 42*). Human lymphoblastoid IF appears similar in this respect to the leukocyte type (*42*).

When these major molecular species of mouse and human IF are separated and tested for their activities, each is equally capable of inducing the priming, toxicity-enhancing, and blocking effects of IF (*21*).

Human fibroblast IF is more "sticky" than is the HuLeIF or lymphoblastoid IF; the latter type has less tendency to be lost through attachment to glass surfaces. If treated with SDS and reducing agents, HuLeIF and human fibroblast IF can be distinguished; even the 2 major molecular species of HuLeIF behave differently in the presence of a reducing agent and SDS. The low molecular weight species is significantly more labile under these conditions (*42, 49*).

The IF also have different binding affinities to various ligands (*50–53*), and these properties have been used in purification schemes. The different molecular species of IF within the same preparation of either human leukocyte or mouse can be distinguished by the range of their activity in heterologous cells (*49, 54, 55*).

It seems clear now that the human leukocyte and fibroblast types are products of different genes. When mRNA was extracted from fibroblasts and translated in heterologous cells (*56, 57*) or in frog oocytes (*13*), the product could be identified as human fibroblast IF. When both fibroblast and lymphoblastoid IF mRNA were translated together in *Xenopus* oocytes, the oocytes translated 2 types of IF, i.e., the total IF activity could be neutralized only by a mixture of antisera against fibroblast and lymphoblastoid IF. In SDS gels, each type behaved true to its origin, and it also did so in its range of cross-species activities (*58*).

The gene coding for the human fibroblast IF appears located on chromosome 9 in man and is on the short arm of this chromosome (Ruddle F: Personal communication). In addition, some clones, which do not contain chromosome 9 but do contain chromosome 5, also produce IF. The same appears true for clones containing only chromosome 2 (*59*). Because each of these genes can be necessary and sufficient for IF production, they are probably structural genes. It is not understood why genes for IF should be on more than 1 chromosome, nor is it known whether the products of these different genes are identical. The genes coding for the HuLeIF and lymphoblastoid IF have not been mapped.

The presence of carbohydrate in IF molecules has been demonstrated directly in SDS gel bands with IF activity that are stainable with Coomassie blue or by the periodate–Schiff reaction (*17, 20*). That IF are glycoproteins has often been postulated also on the basis of the effects obtained through treatment with neuraminidase, acid hydrolysis, and periodate on the molecular weights of the IF (*60*). Mild periodate treatment at low pH can eliminate the charge and size heterogeneities of native mouse as well as HuLeIF and lymphoblastoid IF (*18, 21*). In the latter cases, the larger 21,000-dalton molecules were converted to smaller ones whose activity now focused sharply at pH 5.7. Both effects can also be obtained by treatment with glycosidases (*61*).

It has also been demonstrated that the production of both mouse IF and HuLeIF can be induced in cells in the presence of the glycosylation inhibitors D-glucosamine, 2-deoxyglucose, and Tunicamycin (*62*). Nearly normal amounts of IF activity are produced under treatment with

Tunicamycin, but it virtually all migrates as a band corresponding to 15,000 daltons in SDS gels. This IF also behaves as a 15,000-dalton protein in Sephacryl-200 sizing columns, which suggests that it contains no carbohydrates. Carbohydrate-free IF have been designated as "interferoids," and because they are biologically active for several activities (*21*) and behave like the larger native IF with regard to their hydrophobic properties, one can conclude that the carbohydrate moiety of the native types is not required for either their biological activity or their hydrophobicity.

Those IF believed to be purified to homogenicity have a specific antiviral activity from 2×10^8 (*19*) to 3×10^9 IU/mg protein (*16–18*), which ranks these natural substances among the most active biological compounds known.

BIOLOGICAL ACTIVITIES OF INTERFERONS

The Antiviral Effect

The replication of a wide variety of viruses is blocked in cells pretreated with IF that includes RNA viruses, both single stranded (positive and negative strand) and double stranded, as well as DNA viruses, and lytic and transforming viruses (*6, 63*). Various microorganisms which multiply intracellularly are also inhibited by IF (*64*).

In contrast to the high degree of specificity exhibited by immune defense mechanisms and their action on extracellular viruses, the IF system offers a broad spectrum protection that comes into play during the intracellular multiplication phase of viruses.

The antiviral effect is not exerted directly against viruses but is mediated by cells. This being so, it is the latter, not the viruses, that determine the level of resistance achieved against each particular virus (*65, 66*). Each species of animal and each kind of cell exhibit a particular pattern or relative resistance to different viruses, and some of the latter may therefore appear sensitive to IF-induced resistance in one species and resistant in another. The level of resistance that develops to different viruses varies, as determined by the cell type and the concentration of IF applied to it.

The expression of an IF-induced resistance can follow a short period of IF binding to specific cell-surface receptors. The binding interaction does not require active cell metabolism. Receptors for IF coded for by a gene(s) located on the human chromosome 21 determine the relative sensitivity of human cells to IF action (*3*). Human cells trisomic for chromosome 21 are more sensitive to IF than are cells disomic for chromosome 21, and these, in turn, are more sensitive than cells monosomic for this chromosome (*67*). (For this reason, trisomic cells are often used in assays of human IF.) Binding of an IF to its receptors for as little as 1 minute can trigger the development of the antiviral state, which appears gradually within a period of hours, even in the absence of IF in the medium. However, this process does require RNA and protein synthesis. The antiviral state is maintained in cells for variable periods, measured in hours, and then the cells return to their nonresistant normal condition.

Because the use of antimetabolites (both RNA and protein synthesis inhibitors) can abrogate the development of the antiviral state (*68–70*), it has been postulated that IF induces the synthesis of new proteins necessary for the expression of the induced phenotype, including the antiviral state (*71*).

With continued presence of IF in the medium, the antiviral state can be maintained indefinitely, as are a reduced rate of cell multiplication and other IF-induced effects (*see* below). Therefore, no period of hyporesponsiveness to induction is caused *by* IF. (This is in contrast to the hyporesponsiveness observed upon induction by its inducers.) This fact is significant clinically because it means that the phenotype they induce can be maintained by repeated administration of IF.

However, in its absence in the medium, the antiviral state progressively decays over a period of hours. This decay occurs faster under conditions allowing optimum RNA and protein synthesis (*72*) and can apparently be stopped (*73*) or slowed down (*74*) by the use of antimetabolites. Because the decay is also slower in enucleated cells (*75*), one might conclude that decay of the antiviral state is mediated by a newly synthesized regulatory protein(s) and that, like IF production, it is subject to posttranscriptional control.

The kinetics of induction and of decay of the antiviral state induced by either human fibroblast IF or HuLeIF, as well as by their isolated molecular species (i.e., the 21,000- and the 15,000-dalton components of HuLeIF and the 38,000- and 22,000-dalton components of mouse IF) are similar [(*24*); Stewart WE II: Personal communication]. Also, the relative sensitivities of viruses to each of the human fibroblast IF and HuLeIF are similar in the same cells (*24*), which indicates that the antiviral mechanisms they induce are likely to be the same.

The mechanisms by which IF-treated cells inhibit virus replication are both complex and multiple; much evidence has been accumulated that indicates inhibition of translation of viral proteins does occur (*63, 76, 77*). Others have shown that inhibition of transcription of viral mRNA occurs in polymerase-containing viruses (*78, 79*), perhaps through interference with viral polymerase function. Inhibition at either level can occur in SV40-infected cells, depending on whether IF treatment is given before or after infection (*80*). In C-type oncoviruses, viral inhibition occurs at a late step or at maturation, assembly, or release of virus particles from the cell surface (*81–84*).

In fact, these various mechanisms may all be operative on any given virus, when a multitargeted attack perhaps explains why, although attempted repeatedly, no one has isolated IF-resistant strains of viruses (*85*). This is another observation of consequence with regard to the clinical potential of IF.

Cell-free protein translation systems have recently been used fruitfully by investigators to analyze IF-induced intracellular events. These studies were given much impetus when it was reported that the addition of double-stranded RNA and ATP to saps of IF-treated cells (whether virus-infected or not) greatly activated their translation inhibitory activity (*86, 87*). Inasmuch as the continuous

presence of these factors was not required, it could be concluded that a new translation inhibitory factor had been formed in the translation mix (*88*). This was subsequently confirmed through the isolation of a low molecular weight inhibitor of translation, an unusual oligonucleotide with a 2′–5′ link (pppA(2′p5′A)n (*15*). This compound is synthesized by an enzyme present in extracts of IF-treated cells activated by dsRNA. It can be generated on columns to which dsRNA is immobilized and has also been produced synthetically. An extremely powerful inhibitor of protein synthesis and active at nanomolar concentrations, it appears to act through the activation of an endonuclease (*89*).

These IF-treated cells also reveal 2 phosphorylated proteins in the 30,000- and 60,000-dalton ranges (*90*). They have repeatedly been found in animal and human cells treated with various kinds of IF (*88, 91–93*) and even in virus-resistant mutant mouse cells which are constitutive producers of low levels of IF activity (*94*). Interestingly, in contrast to the relative species specificity of IF themselves, IF-induced activities are not species specific because activated human cell saps can phosphorylate the mouse 67,000-dalton protein marker (*90*), and activated mouse L-cell saps can phosphorylate a protein in the rabbit reticulocyte cell-free system (*95*).

So far, extracts of IF-treated cells have been reported to contain precursors to an endonuclease, a kinase, and a synthetase for the low molecular weight inhibitor of protein synthesis.

In whole cells, a highly selective IF-induced inhibition of the synthesis of viral protein has been described, whereas cellular biosynthetic processes appear unaffected (*76, 96, 97*). However, except in one instance (*98*), this specificity has not been observed in cell-free systems and its mechanisms are not yet understood.

None of the nonantiviral effects of IF has been studied at the subcellular level. Although the present evidence indicates that they are not separable from the antiviral state, whether they are mediated by the same biochemical pathways is not known. Whether or not these pathways are interrelated, IF have pleiotropic activity and are capable, through a single initial interaction with a cell surface receptor, of inducing multiple cellular changes, some of which are common to all IF-treated cells (e.g., the antiviral effect), some particular to certain tissues (e.g., immunomodulation), and others to certain specialized cells (e.g., enhancement of phagocytosis by macrophages).

Cell Surface Alterations

The binding of IF to membrane gangliosides seems to be necessary for the development of antiviral action (*99*). Both cholera toxin and thyrotoxin, which also bind to membrane gangliosides, inhibit the IF-induced development of resistance to viral infection (*100*). Conversely, IF inhibit the binding of thyrotropin to mouse L929 cells and human KB-3 cells (*100*).

Treatment of cells with IF has been reported to 1) enhance the absorption of alloantibody on the surface of leukemia cells (*101*); 2) enhance the expression of H_2 transplantation antigens on thymocytes and splenocytes in vitro and in vivo (*102*); 3) increase the net negative charge of mouse L-cells (*103*); 4) increase the number of intramembraneous C-type particles (*104*); 5) cause alterations in ion fluxes and the electrical potential of cells (*105*); and 6) enhance the excitability of neurons in culture (*106*). Recently, it has also been reported that the total surface of IF-treated, growth-inhibited human fibroblasts and membrane rigidity are increased and that the cell surface distribution of both lectin receptors and LETS protein is altered (Pfeffer LM: Personal communication). These changes induced by IF are "diametrically opposed to the alterations associated with virus-induced cell transformation" (Pfeffer LM: Personal communication).

Cell Structure Alterations

In IF-treated cells, which may exhibit changes in the abundance and organization of cytoskeletal components, the number of actin fibers and microtubules have increased per unit surface area (Pfeffer LM: Personal communication). Such cells are more similar to normal cells than they are to the transformed in these respects.

Inhibition of Cell Multiplication

The multiplication of cells is inhibited by IF treatment. This is true for various mouse cells, e.g., L-cells (*107–109*); L1210 leukemia cells (*110–112*); Friend leukemia cells (*113*); regenerating liver (*114*); and various human cells, e.g., lymphoblastoid cells (*115, 116*), osteosarcoma cells (*117*), human diploid fibroblasts, and HeLa cells (*20, 118*). Treatment also results in lower cell saturation densities in anchorage-dependent cells and in a decreased capacity of transformed cells to form colonies in agarose (*119, 120*). In both respects, IF-treated cells behave more like normal cells. However, they do not appear to be blocked in any particular phase of their cell cycle but to exhibit a lengthening of each phase (*121–123*).

In the HuLeIF and mouse IF preparations, the activity responsible for the inhibition of cell multiplication overlaps with the antiviral activity following fractionation in SDS-PAGE (*112*). Per unit of antiviral activity, human and mouse preparations purified to homogeneity have inhibited cell multiplication as effectively as crude preparations. This finding establishes that cell growth inhibition is an inherent property of the IF molecules themselves (*16, 20, 124–126*).

Some have speculated that the growth inhibitory effect of IF may be important in their antitumor activity.

Priming Effect

Pretreatment of cells with low concentrations of IF may result in an enhancement of its production following induction (*127*), a property that is exploited by IF producers to increase crude yields.

Sensitization of Cells to Toxicity of Double-stranded RNA or to Infection by Certain Viruses

Cells treated with IF are often more sensitive to the cytotoxicity of dsRNA and of vaccinia virus (*93, 128, 129*), provided that the latter two can synthesize new products (*129*). This property is also one of pure IF (*21*).

Immunomodulation

Although IF affect immune functions, timing of the treatment, and perhaps also the dose, determine the kind of effect obtained. B-Cell function can be depressed in vitro and in vivo. Jerne plaque formation and antibody titer of mouse spleen cells to a primary or secondary SRBC antigenic challenge are depressed by IF (*130–133*). The maximum depression is observed, in vitro, when it is added at the same time as the antigen; in vivo this occurs when mice are treated within 48 hours of antigenic challenge. However, when IF is given either 48–72 hours after SRBC (*130–134*) or earlier, but at low concentrations (*135*), an enhancement of antibody response results.

Lymphocyte proliferation is depressed in response to mitogens, as well as allogeneic stimulation, both in vitro and in vivo (*130, 136*) probably because IF inhibits cell multiplication in general. In contrast, it augments the specific cytotoxic response of sensitized lymphocytes (*137*) twofold to fivefold in animals (*137*) and human MLC (*138, 139*), if cells are treated *after* the proliferative phase of the immune response.

Human ADCC responses are significantly augmented by IF (*140, 141*), as is the cytotoxic activity of human NK cells (*142*), which has been reported to occur in animals (*143, 144*) and in patients [(*145*); Chang O; Rasmussen L: Personal communications]. Also, IF treatment enhances the phagocytic activity of macrophages in vitro (*146*) and in vivo (*147*). Mouse peritoneal macrophages are activated in vitro to become cytotoxic for tumor cells (*148*). This effect is depressed by PGE_1 and PGE_2 and by dibutyryl cAMP, which suggests positive and negative controls of macrophage function by IF and prostaglandins (*149*).

Under certain conditions, exogenous and endogenous IF can inhibit DTH reactions (*150, 151*) and either increase (*152, 153*) or decrease GVH reactions (*149–156*). Interestingly, this latter effect is accompanied by a subsequent decreased incidence of lymphomas in graft-bearing mice (*157*). These tumors are believed to be due to the activation of endogenous murine leukemia virus during GVH reactions.

Enhancement or inhibition of graft rejections also seems to depend on the dose given (*158*) and the timing of the treatment. With IF, the possibility of reducing the severity of a GVH reaction in patients who received transplants, a treatment which would double as antiviral prophylaxis, has made this an interesting approach in transplantation medicine.

Blocking

Blocking, like priming, is an effect of IF on its production. When cells are treated for several hours with large doses, they become less capable of responding to an induction for IF production (*159*). Thus far, this blocking effect is the only IF-induced effect observed with impure preparations of this glycoprotein that may not be obtained with completely purified ones (Pitha P: Personal communication).

Other Effects of Interferons

Numerous other effects have been described which consist of either an inhibition, an enhanced expression, or a de novo induction of specific cell products. Tyrosine aminotransferase (*160, 161*), glycerol-3-phosphate dehydrogenase (*162*), and glutamine synthetase (*163*), all steroid-induced enzyme activities, have been depressed by IF. On the other hand, the production of PGE (*164, 165*) and histamine (*166*) are increased in IF-treated cells, as is the production of benzanthracene-induced aryl hydrocarbon hydroxylase (*167*). Thus the IF-induced phenotype is highly complex. The molecular mechanisms of its antiviral state are being studied intensively, but they are still poorly understood, and whether the same mechanisms are functionally related to other aspects of this phenotype is still completely unknown.

ENDOGENOUS VERSUS EXOGENOUS INTERFERONS

Interferon Inducers

Under natural conditions, the most common stimulus leading to type I IF production by an organism is provided by viral infections, specifically by intracellular viral nucleic acid(s). In the laboratory, however, many substances that can act as inducers have been discovered over the years and used experimentally. They belong to a variety of natural or synthetic compounds. [*See* (*1*) for an extensive review.] Their chemical and structural dissimilarities make it difficult for one to determine a unifying concept of the mechanisms of induction (*1*). Different inducers act with various degrees of effectiveness under different conditions, with many being active when injected into animals but not when applied to cultured cells. The nonviral inducer most frequently used under experimental conditions is a synthetic double-stranded poly RNA: poly r(I)n, poly r(C)n, or poly I:C. Discovered in 1967 (*168*), it has been investigated extensively and clinically (*169–171*).

The existence of effective nonviral inducers did indeed raise the hope that the antiviral and other biological properties of IF could be exploited clinically simply through the in vivo induction of the endogenous substance in response to the systemic administration of an inducer.

Whereas several IF inducers, and in particular poly I:C, have shown prophylactic and therapeutic activity against many localized and systemic viral infections, as well as antitumor and immunomodulatory activities in mice (*24, 172*), their clinical potential appears limited. First of all, the repeated administration of any inducer is made increasingly ineffective by the phenomenon of hyporesponsiveness to repeated induction. This has been observed both in vitro and in vivo and to date could not be circumvented, although attempts at doing so are still ongoing (Stringfellow D: Personal communication). In addition, those inducers which appeared most promising from the standpoint of their therapeutic index in animals (ratio of therapeutic effectiveness in relation to toxicity), such as synthetic dsRNA, were surprisingly poor IF inducers in primates, including man; high levels of nuclease activity in primate serum appears to destroy circulating poly I:C rapidly to the marked detriment of its ability to induce

good tissue IF levels. Thirdly, all inducers display toxicity in man, that, at doses which induce therapeutically useful amounts of IF activity, reaches intolerable levels. Thus at least in the clinic, the inducers tested so far have had low therapeutic indexes, and their experimental use has been disappointing (*173*). A nuclease resistant complex of poly I:C with poly-L-lysine, stabilized in carboxymethyl cellulose, has been developed (*174*) that is being tested in clinical trials. It has more promise, both for inducing high levels of IF in primates (*175*) and for having demonstrated *antiviral activity* in such animals (*176, 177*). However, its use may still be limited by hyporesponsiveness to repeated induction (*177*) and probably also by its toxicity [(*178*); Oettgen H: Personal communication].

A continuing search for clinically useful inducers as well as for ways we can circumvent hyporesponsiveness to repeated induction is warranted (Levy HB: Personal communication) but may or may not succeed. Therefore, a renewed interest in the production and use of exogenous IF now exists.

Exogenous IF is that induced in cells either propagated or maintained in cell culture systems. It can be collected, concentrated, and purified to result in preparations which can be injected im, sc, iv, or by other routes. Experimentation with its administration to animals started soon after the discovery of IF. By the early 1960's, researchers had demonstrated that, under appropriate conditions of dosage in relation to kind and dose of virus challenge, IF had a powerful antiviral activity in vivo, both prophylactic and therapeutic, even against highly virulent and potentially lethal infections (*6, 172*). Furthermore, such activity could be demonstrated even through the use of crude, low titer preparations. They were well tolerated by animals even upon repeated high dose administration (*179*). Also early on, human infections, though local, could be prevented (*180*) or treated effectively (*181*) with the exogenous type. Thus, as opposed to their inducers, IF have long been known to have high therapeutic indexes.

The development of IF therapy in man, seriously hampered for a long time by technical difficulties and the high cost of large-scale human cell culture systems required for its production, also seems to have been impeded by the hope generally prevailing throughout the late 1960's and early 1970's that someone would develop a "good" inducer, which would altogether avoid the need to produce IF outside the body. Only after their attempts at using IF inducers clinically proved disappointing did investigators give serious thought once more to therapy with the exogenously produced substance and to large-scale tissue culture systems which could be used as sources of such material.

Large-scale Interferon Production Systems

Such systems have been developed and now function in a number of academic and industrial laboratories that supply IF preparations for experimental and clinical studies. They are of different types designed to accommodate the growth requirements of the various cells used.

Anchorage-dependent Cells

Cells needing attachment to a substrate, such as fibroblasts, are used in production of mouse and human fibroblast IF. Mouse IF, which in early studies was often induced in vivo and extracted from tissues of killed animals, is now mainly and conveniently produced from transformed established cell lines. Anchorage-dependent cells are propagated in culture bottles, i.e., "roller-bottles" (*155*) or their modification "multisurface" roller-bottles. Cells are primed, induced by a virus, and superinduced through sequential treatment with cycloheximide and dactinomycin. Under these conditions, 1 IU of IF activity can be produced/100 cells.

Human fibroblast IF is now similarly routinely produced in roller bottles (*182*). The basic production procedures were developed by Havell and Vilcek (*28*) who demonstrated that selected fibroblast cell strains, derived from human foreskins (such as their FS-3 and FS-4) grown to confluency, aged, primed, induced with poly I:C, superinduced with cycloheximide and dactinomycin, and further incubated for 24 hours, can produce 10^4 IU of IF/ml medium, or about 1 IU/100 cells. Because of the toxicity of the dactinomycin, the cells have to be discarded after superinduction.

Fibroblast IF production for clinical investigations has both certain advantages and disadvantages. The main advantage is that of relative safety for clinical administration because a cell strain can be thoroughly characterized serologically and karyologically for normality. Also, theoretically, virtually no limitation to the amounts of IF that could be produced by cells which multiply in vitro should occur. However, the disadvantages are that normal fibroblasts propagate slowly, they exhibit marked contact inhibition, and they are nutritionally demanding. Moreover, after each production run, they have to be discarded and new cell populations grown, starting with low-passage frozen cell stocks. These characteristics, when compared with those of multilayered transformed cells, account for the costly requirement of fibroblasts for considerable growth surface area per cell, extended time needed to reach confluency, and high content of quality serum in the medium. In addition, priming and superinduction of fibroblast cultures for IF production demand repeated labor-intensive manipulations of culture flasks. Satisfaction of all these requirements results in the high production cost of human fibroblast IF, which, in reality, does limit the amount that can be produced by any laboratory and that can be available for experimental treatment. Despite the fact that several industrial and academic laboratories have undertaken human fibroblast IF production on a large scale, some have closed their operation, and others, still interested in testing fibroblast IF in the clinic, can provide total amounts that are today only about 1% of the leukocyte IF undergoing clinical testing.

A novel approach which should amount to a more economic large-scale culture of anchorage-dependent cells has recently been proposed, i.e., the use of microcarrier beads to which cells attach and which can, in turn, be maintained in suspension (*183*). However, this promising approach is still burdened with a number of technical

problems and probably has not been applied to routine, large-scale, human fibroblast IF production.

Cell Growth in Suspension

Suspension cell culture systems are economical because less medium and less culture vessel volume are required per cell and cells growing in suspension are generally nutritionally undemanding. Certain cells, such as lymphoblasts, grow naturally in suspension, and others can be adapted to do so. All are, however, either transformed or malignant.

For mouse IF production, both an L-cell and Moloney sarcoma virus-transformed 3T3 cells, designated C-243-3, have been adapted to grow in suspension (*184*) and can produce IF in fermentor-type containers where optimum culture conditions are maintained automatically (*10*).

For the human product, suspension cultures have the potential of being economically the most advantageous system. Human lymphoblasts containing an integrated EBV genome or parts of it are capable of rapid and continuous growth in suspension. A number of such cell lines have been isolated and tested for their ability to produce IF. Some of them were actually found to be constitutive producers of various amounts of the leukocyte type (*185, 186*), but no correlation is apparent between the level of expression of the EBV genome and the level of IF spontaneously produced. Although no exhaustive screening of all human lymphoblastic cell lines has been done (1982) for their relative efficiency as IF producers, the Namalva cell line, derived from a patient with Burkitt's lymphoma, has been chosen from among those few tested for giving good titers when stimulated with Sendai or Newcastle disease viruses (*61, 187*). It is presently being grown in large fermentor-type vessels at the Wellcome Laboratories in England and is used for human IF production at the pilot plant scale. Optimum culture conditions are maintained automatically. Cells are primed, virally induced, incubated for production in low-serum content medium, and discarded. Although crude IF titers in the medium are not high (1 IU/1,000 cells), unit cost is sufficiently low when the system operates smoothly, to make it, under these conditions, the most economical production system developed so far.

However, this system also has some disadvantages, the major one being that lymphoblastoid IF is the product of malignant human cells.

Short-term Maintenance Cultures

A few investigators deliberately concentrated on developing a system allowing the large-scale production of a human diploid cell IF under conditions that would be cheaper and simpler than fibroblast IF production. When nucleated cells from peripheral human blood were separated from RBC and maintained in growth medium, they could be induced to produce an IF (*188*). The use of such cells avoided one of the main problems in human fibroblast production, i.e., the necessity of continuous growth of large numbers of nutritionally demanding cells from low passage frozen cell stocks.

Peripheral blood WBC were then and still are the cheapest normal nucleated human cells that can be obtained in large quantities.

Cantell of the State Serum Institute in Helsinki, Finland, has developed (over years of efforts) a set of procedures allowing the use of human peripheral WBC as a source of substantial amounts of a leukocyte IF preparation for clinical use. Through an active collaboration with the Blood Transfusion Service of the Finnish Red Cross, he also built the first sizable production center for such IF that has existed worldwide.

Technicians in modern blood centers routinely process blood to separate packed cells from plasma and platelets. As a component of blood collected for transfusions, leukocytes are clearly undesirable. Under appropriate conditions, a single centrifugation run can separate a leukocyte-rich cell layer (the buffy coat) between the heavier packed RBC and the lighter platelet-rich plasma. Buffy coats can be harvested with only insignificant losses to the plasma and RBC fractions. These can still be used, and the cost of separating the buffy coats, each of which contain on the average 10^9 or more WBC, amounts almost solely to that of the minimal additional handling required for their separation.

All blood donations are routinely tested for sterility and the presence of hepatitis B surface antigen (HbsAg) within a few hours of collection. Because buffy coat cells remain susceptible to induction for IF production for more than 24 hours, contaminated buffy coats can be identified and discarded. Those pooled for IF production are treated with an ammonium chloride solution to lyse the RBC, and the leukocytes are collected in a continuous flow centrifuge. These cells are counted and suspended in a culture medium at a cell density of 10^7 cells/ml in which they are primed with a low concentration of IF and infected with Sendai virus (a *p*-influenza 2 virus). The cell concentration is adjusted to 10^6 cells/ml in growth medium supplemented with 4% human serum, and cells are incubated as a short-term suspension culture for 18–24 hours, during which IF biosynthesis and secretion occur. The cells are then separated by centrifugation and discarded. The culture medium, which constitutes the crude IF, reaches titers of 10^4 or more U/ml. Each buffy coat yields approximately 2×10^6 U of crude IF.

The simple and relatively effective method of purification developed by Cantell involves precipitation by potassium thiocyanate and solubilization of IF in acid alcohol, from which many impurities are gradually eliminated by reprecipitation when the pH is slowly raised. The IF starts precipitating at pH 6 and is recovered by centrifugation and dissolution in PBS. A 50- to 100-fold purification is achieved with recovery of about 50% of the crude activity. The specific activity of the partially purified IF obtained (designated by Cantell as "P–I") is from 10^6 to 10^7 U/mg of protein. This preparation does not contain live Sendai virus (which is inactivated by the acid ethanol step) and is stable (*121, 189*).

From the standpoint of unit cost of production, leukocyte IF production is costlier than that of lymphoblastoid but less expensive than production of fibroblast IF; it is certainly simpler than either of them, particularly for laboratory staffs who have to start "from scratch."

For the purpose of rapidly and substantially building up

the total pool of human IF available for clinical trials, Cantell's method appears to offer, at present, the method of choice if one considers all pertinent factors: ease and possible rapidity of implementation; low starting cost; acceptability of the product by the Bureau of Biologics (now the National Center for Drugs and Biologics, Food and Drug Administration) for investigations in human subjects; and demonstrated effectiveness of the leukocyte IF in man.

PHARMACOKINETICS OF INTERFERONS

Following systemic administration, IF have a short half-life in circulation. Because little is excreted in the urine, it is assumed that they disappear from blood circulation through becoming either diffusely bound or inactivated in tissues, or both. The pattern of IF clearance is rapid at first and then slower, and this pattern has been observed in all species studied. In clinical studies done with leukocyte IF, the most rapid clearance is noted following iv inoculation; im injection gives longer lasting levels of circulating IF (peak blood levels are attained 2–3 hr after injection and maintained for 4–6 hr), and clearance is even slower after sc inoculation. With im doses of 2×10^5 U/kg body wt, one can maintain about 100 IU of IF activity/ml of plasma for about 12 hours (*32, 190*). To prolong this plateau phase, one can only resort to giving a higher initial dose (*191*). The plateau or slower clearance phase is believed due partly to the slow release of previously tissue-bound IF.

Inasmuch as similar levels can be found throughout most tissue following an inoculation of exogenous IF, it is apparent that these substances can diffuse rapidly into tissues and organs. At least one important obstacle to such diffusion is known, i.e., the blood brain barrier (*192*). Intrathecal inoculation has been tried in a few patients, including those with herpetic encephalitis [(*193*); Merigan TC: Personal communication] suspected rabies, and in 1 leukemic child (Hill N: Personal communication), without apparent untoward effect on them.

The above observations were made in humans treated with leukocyte IF. Recently, some have recognized that, following fibroblast IF administration, the disappearance of activity from the plasma is much more rapid (*194, 195*); IF activity becomes undetectable within minutes of such an im inoculation, even after doses similar to those resulting in a long-lasting plateau with leukocyte IF. The rapid clearance of human fibroblast IF clinically is in fact similar in duration to that of mouse IF in the mouse (*196*). In addition, it now appears that both forms are susceptible to certain inactivating factor(s) present in vivo (*197*), particularly in muscle tissue extracts (*198*). To what extent, if at all, this phenomenon affects the therapeutic value of fibroblast IF is not known because the very significance of IF serum levels is not understood either, inasmuch as the latter measures only unbound and perhaps useless IF. In a preliminary way, however, it is generally agreed that therapeutic effects achieved by these exogenous or endogenous glycoproteins do correlate with high and lasting levels achieved in the circulation and in tissues. For this reason, perhaps fibroblast IF should be administered iv rather than im for systemic treatment. We need many more pharmacokinetic studies in man and animals to clarify the many questions regarding dosage and route of administration as well as possible different tissue affinities by different types of IF (*199*).

Similarly, the possible side effects of treatment with IF need further study. Because they are physiological substances, it is not surprising that they do not cause symptoms of gross toxicity. At doses active in man in antiviral and perhaps also antitumor therapy, primarily transient low fever, fatigue, and redness at the site of injection have been reported in treated patients (*30, 32*). Upon daily treatment with what is considered today at high dose, such as 10^7 U or more, moderate hair loss has been observed occasionally, as well as leukocytopenia and granulocytopenia (*190, 200*) and reduced platelet counts (*190*). However, WBC and platelet counts appear to stabilize at lower levels without apparent adverse effects, and dose reduction has not been necessary during prolonged treatment except in severely immunosuppressed patients (Hirsch M: Personal communication). Transient elevations in hepatic enzyme activities have also been reported (*30, 201*). All these effects appear mild and all are transient; values return to normal upon cessation of IF treatment. Also, the incidence and intensity of these manifestations appear to decrease as the purity of the preparations increases (*202*).

In the mouse, experiments have been done with high dose administration for exploration of the toxic range of dosage in that species. Newborn mice given daily injections of 50,000 U of mouse IF failed to gain weight normally and died during their second week of life showing extensive liver degeneration and other disorders. (This size inoculation in a mouse weighing 1 g is the equivalent of 5×10^7 U/kg body wt. Such a dosage would amount to a daily administration of 3.5×10^9 U in an adult human being. The dosage used presently in man is in the range of $0.5–2\times10^5$ U/kg body wt-day.) If treatment was stopped after 1 week, most of the mice survived at first only to die starting 1 month later with severe glomerulonephritis (*203, 204*). These experiments were done under conditions which clearly impute these effects to IF, and, although done in newborn animals with daily doses per unit weight 100–1,000 times higher than have ever been given to patients so far, their results warrant attention.

As has been pointed out (*64*), IF are capable, if not of outright toxicity, at least of profound modifications of numerous cell functions and, therefore, presumably also of altering subtle relationships between cells, tissues, and organs. The outcome of such alterations may be, if not injurious to the cells, injurious to the organism as a whole.

PROPHYLACTIC AND THERAPEUTIC EFFECTS OF INTERFERONS

The account and discussion of their prophylactic and therapeutic effects will cover studies in humans and in animal model systems. For brevity, we will concern ourselves only with the administration of exogenous IF. Even so, the number of pertinent studies done in the course of the last 20 years is large, and only illustrative examples, rather than an exhaustive account, can be given here.

Interferons as Antivirals

In Animals

The scarcity and low titers of the early preparations used in any in vivo studies, as well as a lack of understanding of viral pathogenesis and of the stages in these pathogenic processes when IF treatment could be expected to have an effect, account for the number of early investigations that resulted in either negative or inconclusive results [for review, *see* (*6*)]. This applies particularly to therapeutic, as opposed to prophylactic, effects in animals. Nevertheless, once investigators become aware of the fact that dose and time of IF administration must be related to kind and amount of virus in an inoculum, it also became progressively clear that not only prophylaxis of viral infections but also therapy could be achieved with IF treatment and that this could be so even in systemic potentially fatal diseases (*205, 206*). This evidence is now conclusive for diseases caused by both lytic and oncogenic RNA and DNA viruses (*64*). Infections effectively prevented and/or treated with IF were caused by the following viruses: Sindbis and Semliki Forest, 2 togaviruses (*205–207*); the rhabdoviruses, vesicular stomatitis (*208*), and rabies (*209–213*); a picornavirus and an encephalomyelocarditis virus (*214, 215*); vaccinia virus (*216*); the Gross oncovirus (*217*); Friend (*218–222*); Rauscher (*223–226*); RaLV; radiogenic leukemic (*227*); Harvey strain murine sarcoma (*228*); Rous sarcoma (*229*); the DNA tumor; and polyoma (*230*). In most of these infections, viral parameters in addition to pathogenesis were followed, and IF administration either aborted infections if given before the challenge virus or limited its spread, and a clear therapeutic effect was achieved in the course of an otherwise progressive disease.

In Man

The clinical use of IF has been and still is severely restricted by the scarcity of appropriate preparations. The little there has been has logically been applied first to localized infections in topical administration. As early as 1962, a well-controlled experiment was performed with volunteers undergoing vaccination against smallpox. It demonstrated that a minute amount of locally applied IF produced in monkey cell cultures (primate IF are active in human cells) could effectively inhibit vaccination "takes" (*180*). At about the same time, it was demonstrated that vaccinial keratitis could be treated effectively in man [(*181*); also with monkey IF] as it had been in rabbits (*216*). Thus since 1962, we have known that IF treatment could have both prophylactic and therapeutic antiviral actions in humans.

Topical treatment of viral infections with human IF was later tried in the respiratory mucosa (*231*) and the corneal epithelium (*232, 233*), respectively, against myxoviruses and herpes simplex virus. Each of these trials was done under fully controlled conditions, and those involving herpetic infections of the eye were not done in experimental infections but for the first time in a natural disease. They all demonstrated clear therapeutic effects.

The scarcity of human preparations has made it impossible for investigators to attempt the primary or secondary prevention of viral infections in man, although, on the basis of work in animals, IF is likely to be most effective when used in this way. Administration in the postinfection but presymptomatic phase of human rabies, e.g., would quite certainly have life-saving effectiveness, as has been demonstrated repeatedly in animals (*212, 213, 234*). The experiment must wait for more human IF to be available because fatal CNS infection occurs only in a proportion of those bitten by rabid animals, and a substantial number of infected, but not symptomatic, people would have to be treated for a demonstration of an unequivocal prophylactic effect.

However, evidence for IF-mediated protection against viral infections was derived from observations made in cancer patients included in trials. Some of them have been treated and later followed for long periods. Despite the fact that cancer patients are notoriously prone to diseases caused by viruses (*235, 236*), particularly when receiving immunosuppressive treatment (*237, 238*), the clinical impression is that those receiving IF may have a lower incidence of viral infections, or may cope with viral infections more effectively, or both [(*30, 239, 240*); Gutterman J: Personal communication]. These observations suggest that IF treatment offers effective protection against systemic and localized viral diseases in cancer patients.

Systemic administration of IF for antiviral therapy has been attempted only recently. In the three diseases in which it has been tried so far, chronic active hepatitis B (or chronic serum hepatitis), herpes zoster, and varicella in children, the results have been positive.

Chronic active hepatitis B was selected for trials because several viral markers can be quantitated in the patients' sera which allows an objective evaluation of the antiviral efficacy of the treatment. (The markers are HbsAg, HbcAg, the viral enzyme DNA-polymerase, a viral dsDNA, and a soluble antigen e, associated with transmission of the disease. Usually, patients also have a high antiserum titer against the HbcAg and some have circulating Dane particles, believed to be the viral etiologic agent.) In one trial (*190*), 4 patients were studied, each of whom had high circulating levels of virus markers for more than 6 consecutive months. Each injection of IF elicited a pronounced and dose-related response, particularly evident on DNA-polymerase levels. Other markers decreased only upon repeated daily injections. If IF was given for 10 days or less, its effects were transient; upon more prolonged treatment, they became longer lasting, persisting for several months beyond the treatment period.

Early intensive high-dose IF therapy ($>10^7$ U/day) followed by maintenance therapy at low doses (10^6 U/day) has now resulted in suppression of all virus markers and progressive improvement in liver functions, which indicates that the eradication of the virus may be possible (Merigan TC: Personal communication). Two other studies, one in man with poly I:C-induced fibroblast IF (*241*) and the other in chimpanzees with the inducer polyriboinosinic-polyribocytidylic acid poly-L-lysine (*177*), confirm the sensitivity of Dane particle markers to IF treatment.

Chronic hepatitis B_x a disease for which no effective treatment is available now, is the most important viral disease, the incidence of which is on the increase in the western world. It is prevalent in developing countries where

as much as 15–20% of the population may be chronically affected. Chronic cases arise in about 10% of those suffering at any one time from acute hepatitis, and those patients with the chronic *active* form are not only carriers, but contact with them would be contagious. An antiviral treatment which can suppress the infectivity of hepatitis virus carriers also offers the hope that the disease itself might one day be eradicated (*242*).

Controlled clinical trials of IF in 90 cancer patients with herpes zoster disease (shingles) have followed encouraging results obtained in earlier, noncontrolled clinical investigations (*32, 243*).

The IF system appears to play an important role in the natural defenses against the varicella zoster virus. Endogenous IF can be detected in the skin vesicles of patients with chickenpox and herpes zoster. Patients whose vesicles have high IF titers only occasionally develop disseminated disease and heal more rapidly; such titers are the only variable with prognostic value (*244*). The positive results of the prospective, randomized trials with patients with herpes zoster establish unequivocally the therapeutic effect of IF in this disease. Acute herpetic neuralgia subsides significantly faster, cutaneous lesion dissemination is lessened, and visceral complications and the usual resulting mortality (not uncommon in cancer patients) are significantly decreased (*245*).

The use of IF is also being tested for the prevention of herpes virus reactivation following surgery for trigeminal neuralgia (tic douloureux of the face) believed to be due to herpes labialis infection, with latent virus in the trigeminal ganglion. A statistically highly significant difference has been found between IF- and placebo-treated patients in the frequency of virus isolation and reactivation following surgical stimulus (*246*).

Because of herpes virus sensitivity to IF therapy, a trial has been undertaken in leukemic children with varicella or chickenpox. Usually chickenpox is an innocuous disease resulting from a primary infection with varicella virus, the same virus which years later may reappear to cause zoster. However, in leukemic children whose immune defenses are often seriously impaired, varicella is a potentially fatal disease. Promising preliminary results with IF therapy have been published (*247*).

Newborn infants with CMV infections have been treated experimentally with IF. The early attempts made with low titer preparations were disappointing (*248*). Later more encouraging results were obtained because excretion of the virus in urine could be suppressed (*249, 250*). Presently, it seems clear that congenital CMV infection is controllable by IF therapy (Merigan TC: Personal communication); this is most encouraging because the cytomegalic inclusion disease of the newborn can be fatal (*251*) and be associated, in those untreated and chronically infected infants who survive, with brain damage, mental retardation, and serious physical defects (*252, 253*).

With CMV infections of major importance in transplantation medicine, the virus is the single most important microbial pathogen in renal transplant patients, who often succumb to CMV-induced hepatitis, pulmonitis, or mononucleosis. It also induces immunosuppression and leukopenia, which are associated sometimes with fatal bacterial and fungal infections. Two double-blind, placebo-controlled trials have been conducted with fibroblast or leukocyte IF in renal transplants. Although fibroblast IF failed to prevent viral infections in renal allograft recipients (*254*), the incidence of CMV excretion and viremia, as well as those of herpes simplex virus and EBV, were significantly decreased in patients treated with leukocyte IF. It appears that such treatment can have significant prophylactic efficacy when administered during the posttransplantation period (*255*).

Interferons as Inhibitors of Intracellular Parasites

Few studies have been done on the effects of IF on the growth cycle of intracellular parasites, and most of these were not done in vivo. However, IF can inhibit *Chlamydiae* in cell cultures and in eggs (*256, 257*). *Chlamydiae,* which cause important infections, such as psittacosis, are closely related to the agents that cause eye diseases, including trachoma, which, with an estimated 500 million victims, is the world's leading cause of blindness.

Several parasitic protozoa (toxoplasma, sporozoa, trypanosomes, and plasmodia) have been studied for their susceptibility to IF. *Eimeria,* a parasite of birds (*258*), and *Eperythrozoon coccoides,* a parasite of rodents (*259*), are susceptible during their intracellular growth phase in vivo. Both IF and its inducers have been strikingly effective in inhibiting the outcome in the mouse of infections with sporozoites of *Plasmodium berghei* (*260–262*). Similar studies with trypanosomes are inconclusive (*258, 263*). However, the antiparasitic effects observed were always clearly mediated by cells, as are the antiviral effects of IF.

Given the extraordinary public health and economic importance of many diseases caused by intracellular parasites, the latter organism's susceptibility to inhibition by IF should have more than academic interest.

Interferons as Antitumor Agents

In Animals

Different kinds and degrees of purity of IF preparations can exert antitumor effects in various types of tumors:

Experimental virus induced.—Antitumor effects have been obtained with the use of Friend leukemia (*218–222*), Rauscher leukemia (*223–225*), and Moloney (Harvey strain) sarcoma (*228*) of the mouse, Rous sarcoma in the chicken (*229*), polyoma virus-induced tumors in the hamster (*230*), Shope fibroma of the rabbit (*264*), herpes virus Saimir-induced lymphomas in the marmoset (*265*), and the owl monkey [(*266*); both of the latter with HuLeIF].

Negative findings, i.e., no effect on Rauscher and Friend virus-induced leukemias, were also reported (*267, 268*). Gazdar (*269*) reported tumor enhancement following a single high dose of IF before tumor virus inoculation that could have been due to an immunosuppressive effect by the IF on the primary immune response to the virus.

Possible mechanisms of action in the above tumors appear to vary. When viral oncogenicity is proportional to the size of inoculum, such as with Rous, polyoma, and Shope viruses (cells become transformed but are nonproducers), an early virus-dependent event is likely to be

inhibited by IF. As in vitro, IF probably inhibits cell transformation, which results in a reduction of the number of tumors and an increase in the number of tumor-free animals. In such cases, IF given just before, with, or immediately after virus challenge can be effective (*230, 264, 267*).

With Friend and Rauscher viruses, however, only when IF treatment was given at high doses after virus inoculation and for prolonged periods was tumor appearance delayed and tumor development inhibited. No tumor regressions were reported, and it is likely that, upon stopping treatment, all animals still develop tumors [for a more thorough discussion, *see* (*32*)].

Spontaneous.—The AKR leukemia (*32, 270–274*) and spontaneous mammary carcinoma [(*217, 275, 276*); Bekesi JG: Personal communication] of the mouse were treated with IF with results as discussed below.

Many investigators have used the AKR leukemia to evaluate antileukemia immunotherapy and chemotherapy. The results obtained with chemotherapeutic drugs have generally been predictive of the drugs' effects in man (*277*). Just as in the experimentally induced leukemias, IF had to be given at high doses ($1–2\times10^4$ U/day) for long periods. In preleukemic animals, treatment for 1 year prolonged survival by about 100 days, and the total incidence of leukemias was reduced from 95% in control mice to 63% in IF-treated animals (*272*). In 1 study, treatment was started late in the preleukemic stage, i.e., at 6–8 months of age, and still achieved an inhibition or delay in tumor appearance (*273*). With extremely high doses or 10^5/day, tumor regressions were reported by 1 investigator (*273*) but not by Gresser (*147*). However, under these conditions, Gresser observed doubling (or 100% increase) of life-span, even when treatment was started after a clinical diagnosis of leukemia. Such an effect can be achieved with only 4 of 27 active chemotherapeutic drugs (*277*) and represents an antitumor effect obtained by about 0.1 μg IF protein/day on a tumor load estimated to be approximately 10^9 cells (*147*).

The effects obtained in spontaneous mammary carcinoma of the mouse were a delayed appearance of tumors and a slowing down in tumor progression with a resulting modest increase in life-span (an increase of 25 to 33%). When these studies were done, it came as a surprise that no decrease in gs protein was observed. Today, knowing of the special antiviral effect IF exerts on oncoviruses, i.e., an inhibition of viral assembly or release but *not* of most viral protein synthesis (*81–84*), these observations are interpretable. The fact that extracellular oncoviruses are suppressed as long as IF is present but are released, perhaps in a "burst," as soon as it is removed, explains also why antitumor treatment with it in mouse leukemias and spontaneous tumors of viral etiology has to be at high doses and continuous throughout the life of the animal to be effectively protective.

Transplantable.—These have been the EA, EL 4, L1210, and RC19 of the mouse that were extensively studied by Gresser and his group (*278–283*). Following inoculation of 10^4 EA cells, mortality was 100% at day 22 in controls, but 90% of IF-treated animals survived more than 6 months (*279*). Standard treatment was about 20,000 U daily for several weeks, and appropriate controls demonstrated conclusively that the active substance was IF.

Gresser also studied Lewis lung carcinoma of the mouse, a metastasizing tumor usually resistant to therapy (*284*); IF was active against both primary tumors and lung metastases (*285*).

Transplantable mouse tumors, studied by others and found susceptible to inhibition by IF, were: Ehrlich carcinoma (*286*), Ehrlich ascites (*287*), L1210 leukemia (*287*), sarcoma 180 (*288*), polyoma-induced sarcoma (*289*), Friend leukemia cells (*290*), and osteosarcoma (in which IF was again active against both primary tumors and lung metastases (Glasgow LA: Personal communication). In other species, the Brown Pearce carcinoma of the rabbit (*291*) and the Walker carcinoma of the rat (*292*) were also susceptible to it.

Negative results were reported with L929 cells transplanted in the mouse (*293*). However, treatment was with 1,000 U given three times per week, i.e., considerably less than was used in experiments showing positive effects (usually $>$ 10,000 U/day were given).

Few studies have been done with Type II mouse IF preparations. Type II had more powerful antitumor effects than did Type I. When 300 U of Type II were injected daily directly into sarcoma MC-36 tumors, or 600 U were given systemically daily to mice with metastatic osteosarcoma (Glasgow LA: Personal communication), positive results were comparable to those obtained with over 10^4 U of Type I also given every day. However, Type II preparations are rich in various lymphokines, and their activity cannot be attributed to this IF alone. However, these observations warrant intensified work with production, purification, and characterization of Type II IF preparations (*294*).

Malignant cells of human origin, as learned from personal communications with several investigators, i.e., HeLa (Taylor-Papadimitriou J), bladder cancer (RD 4 line; Horoszewicz J), mammary carcinoma (Taylor-Papadimitriou J), melanoma and osteosarcoma (Kishida T) cells, transplanted sc into nude mice were susceptible to inhibition by *human*, not mouse, IF, which suggested a direct, non-host-mediated effect.

The appearance of tumors following inoculation of transplantable tumor cells and their progressive growth are due to tumor cell multiplication and not to tumor cell recruitment through virally induced, host cell transformation. The mechanism of the antitumor effect must therefore be different from that assumed to be operative in mouse leukemias.

Chemically induced.—In the mouse, IF treatment has inhibited tumor induction by MCA, and tumor development was either delayed (*295*) or completely inhibited in most animals (*296*).

Radiation-induced.—Low dose, but continuous, treatment, started immediately after X-irradiation, significantly decreased the incidence of radiogenic lymphoma in the mouse (*297*). Because radiation induced an endogenous C-type virus, this effect may be due to an antiviral effect.

Combination antitumor therapies in animals.—Synergistic effects have been reported following IF therapy combined with the use of various cytoreductive methods.

In the first such observation, more than 70% of the mice

inoculated with LSTRA leukemia and treated sequentially with BCNU and IF became long-term survivors, whereas IF alone had no measurable effect, and BCNU alone produced only 25% long-term survivors. Changing the order in which the treatments were given abrogated the effect. In this experiment, long-term survivors could live beyond 90 days. When killed at 90 days, no leukemia cells could be found in them (*298*).

The treatment of overt AKR leukemia with CPP, vincristine, or Cytoxan followed by IF has also resulted in much more prolonged survival than after either chemotherapy or IF alone (Bekesi JG; Chirigos M: Personal communications).

Comparable results were obtained in a solid tumor, a transplanted mammary carcinoma, with the combination of adriamycin and IF (Bekesi JG: Personal communication). Similarly, when mice underwent surgical resection of a primary mammary carcinoma, their life-spans were increased by 79%. When such surgery was followed by IF treatment, survival was increased by 215% (Bekesi JG: Personal communication). Even the use of the mild immunostimulant isoprinosine (with no effect on transplanted sarcoma 180 cells alone) before IF treatment resulted in a doubling of the number of long-term survivors among the treated, sarcoma 180-bearing mice (*299*).

The above antitumor effects can be attributed to IF because: 1) A dose–response correlation was observed in several systems [(*147, 217*); Glasgow LA: Personal communication]. 2) Treatment with anti-IF globulin caused marked tumor enhancement in mice infected with Moloney sarcoma virus (*8*). Tumors appeared earlier and were much larger in animals so treated. The tumor-inducing potential of Moloney sarcoma virus was increased 100 times in anti-IF-treated mice. The same was observed in Rauscher leukemia virus-infected mice (*300*). 3) In a syngeneic transplantable mouse tumor, completely purified mouse IF protein with an activity of 2.4×10^9 IU/mg protein inhibited tumor growth to the same extent, unit per unit, as partially purified or crude IF (*16, 301*).

Possible mechanisms of antitumor action.—Like the antiviral activity of IF, its antitumor activity appears effected through several mechanisms. Some seem to act directly on tumor cells and others to be mediated by the tumor-bearing host.

In virally induced tumors, nonproductive, transforming cell–virus interactions require that IF be administered before or simultaneously with the viral inoculum. The inhibitory mechanism is clearly antiviral; it interferes with an early event required for cell transformation. In virally induced or spontaneous tumors releasing transforming virus particles, in which repeated cycles of virus multiplication and shedding occur throughout the animal's life-span, IF shows an antitumor effect only when administered at high doses, daily, and over long periods. Its main benefit may be in reducing viremia (*217*). In both types of viral oncogenesis, the antitumor effect seems to be mediated principally through an antiviral effect. In Friend leukemia, an inhibition of cell multiplication probably also plays a role (*290*), as well as an antibody-dependent cytotoxic immune reaction (*302, 303*).

The importance of an antiviral effect in this kind of system has been demonstrated by the use of an anti-IF globulin, which greatly potentiated the tumorigenic potential of Moloney sarcoma and Rauscher leukemia viruses (*8, 300*).

Transplantable tumors have been useful in predicting the effectiveness of potential antitumor drugs in man. In addition to IF concentration, tumor load, as well as an intimate contact between the IF and the tumor cells (such as when both are injected ip), are important factors that determine its effectiveness against transplantable mouse tumors. Given before the tumor cells are inoculated, IF is inactive; it must be given *after* cell inoculation. Only Graff et al. (*273*) reported evidence of cell killing, although the number of tumor cells that can be recovered from the peritoneal cavity of treated animals on days following cell inoculation is significantly reduced (*282*).

Direct effects on tumor cells appear involved, perhaps through an inhibition of cell multiplication, or cell surface effects are involved, as have been observed repeatedly in vitro. The tumorigenicity of IF-treated cells appears reduced, a finding observed in association with increased contact inhibition and an inability to form colonies in semisolid media, induced by IF in vitro (*119, 120, 295, 304, 305*). Cultivation of mouse L1210 cells in the presence of mouse IF for as little as 24 hours was associated with over a 100-fold decrease in tumorigenicity of these cells (*305*).

Alterations in the expression of cell surface antigens, such as an increased expression of tumor antigens (which has not been determined), might make tumor cells more susceptible to elimination by the host. Good evidence indicates that host-mediated mechanisms are also at play. When mice were inoculated with L1210 cells resistant to the antiviral and growth inhibitory actions of IF (L1210*R* were selected in vitro in the continuing presence of IF), these cells were nevertheless susceptible to its antitumor activity in vivo. When recovered from animals and recultured in vitro, they had maintained their characteristic resistance to the antiviral and growth inhibitory effects of IF (*295, 302*). Perhaps particularly relevant to a host-mediated antitumor effect are its ability to enhance the cytotoxicity of sensitized T-lymphocytes (*137–141*), the activity of NK cells (*142–145*), and phagocytosis by macrophages (*146, 147*). All these effects have been observed both in vitro and in vivo. In short, IF may be able to slow tumor cell growth and normalize tumor cell behavior, or it could increase the "foreignness" of tumor cells and the ability of host cells to recognize them as such and destroy them.

Other mechanisms may be at play. Tumor cells secrete various factors, which are immunosuppressive or stimulatory of mitotic activity. Because it can modulate the expression of cellular genes, IF may also inhibit the biosynthesis of such factors by tumor cells or modify the hosts' cells sensitivities to them. Further research is needed to clarify these important questions [for further discussion, *see* (*64, 147*)].

Potential establishment of treatment regimens for humans by animal experiments.—In the above descriptions and discussion, no specific doses and regimens of adminis-

tration have been related, except in the most general terms, such as high versus low dose, continuous versus short-term treatment, and the like. Extrapolation of IF dosage from animals to man is not warranted except to provide the broadest frame of reference.

Units of mouse L-cell and human IF are not equivalent. They are different molecules, produced by different types of cells stimulated by different inducers; moreover, human and mouse IF are tested for activity on different cells, with different viruses, i.e., in systems that perhaps vary in sensitivity. It has recently become apparent that mouse L-cell IF and HuLeIF have different clearance rates in vivo and perhaps different biological activities as well. For example, with a single exception, no tumor regression has been reported after mouse IF was administered, even at doses of over 10^5 mouse U daily; however, such high doses seem to produce a profound immunosuppression (*217*).

In a 10-g mouse, they correspond to over 10^5 U/kg body wt. Unit per unit, the doses of human IF that have resulted in tumor regression in man are about 100 to 1,000 times lower. The HuLeIF that has produced these effects showed a slow clearance rate in vivo, whereas that of mouse L-cell IF is extremely rapid, as is that of the human fibroblast product. Slow clearance may be important in itself for efficacy, but this remains to be determined in future studies.

The etiology of mouse tumors is, in many cases, different from that of human tumors. Only radionuclide- or carcinogen-induced tumors of the mouse may be good models of human cancer and perhaps transplanted syngeneic tumors with a low degree of malignancy.

In summary, the following can be learned from animal antitumor studies:

1) The IF from several species appear to have antitumor activity upon systemic administration, but only the mouse has been studied in some depth.

2) The mouse IF does not seem to kill tumor cells directly either in vitro or in vivo, and, although tumor cell killing activities are enhanced by IF in vivo, massive tumor cell death in IF-treated animals has not been observed.

3) For optimum antitumor effectiveness, mouse IF has to be administered in such a way as to be in intimate contact with tumor cells, perhaps because it clears rapidly in vivo.

4) It cannot be used prophylactically, not even shortly before transplantable tumor cell inoculation but must be used therapeutically and repeatedly.

5) Optimum doses are apparent below which less than optimal or no antitumor effect occurs, and above which an immune suppressive effect may negate treatment benefit.

6) Treatment has to be prolonged for prolongation of survival.

7) The effect obtained is inversely proportional to tumor load.

8) Tumor load can effectively be reduced with cytoreductive chemotherapy or surgery, which seems to result in more than an additive benefit.

9) The IF system may be an effective defense system against tumor cells independently of the immune system, although it functions better in conjunction with an intact immune defense system.

10) Long-term IF therapy appears to be tolerated well and does not seem to have long-term unfavorable side effects.

11) At high daily doses and at early stages of development, it can have unfavorable, even lethal, side effects.

12) From the pharmacological standpoint, mouse L-cell IF appears similar to human fibroblast IF, and it may share some of the latter type's less favorable characteristics of instability in vivo.

13) To the best of our knowledge, the mouse does not produce the equivalent of HuLeIF, not even from its leukocytes, and one should not extrapolate in any rigid way from the mouse to the human species in planning human trials with HuLeIF or the lymphoblastoid type.

14) In response to antigenic or mitogenic stimuli, mouse lymphocytes produce, as do human lymphocytes, an acid-labile lymphokine with IF properties which, in a still impure state (1 ppm purity) has a considerably higher ratio of antitumor and antiviral activity.

15) Under the most favorable conditions of antitumor treatment, such as optimum dose and extremely low tumor load of transplantable tumor cells (even if highly malignant and representing several hundred LD50), or in combination therapy with effective cytoreduction, in a few experiments IF treatment has either completely prevented appearance of tumors or achieved long-term survival approaching or equaling normal life-span.

16) Knowledge is still fragmentary and much more animal experimentation should be performed on the pharmacology of animal IF, their mode(s) of antitumor action, and IF therapy studies in better models of human cancer.

In Man

Most of the data summarized below were reported during the Second International Workshop on Interferons held in New York in April 1979. (*See* Section II for updated results.)

In all attempts to demonstrate antitumor activity with systemically administered human IF, investigators have used the leukocyte form.

The product made from human fibroblasts, which is even more scarce, has been used mainly for inoculations into or near superficial tumor nodules.

The lymphoblastoid IF and Type II, which cannot be produced in large amounts, have not been evaluated in humans as of this writing (September 1979).

Fibroblast interferon

Human fibroblast IF has been injected at 0.5×10^5 U/day for 30 days into superficial nodules metastatic from melanoma, breast carcinoma, and prostate cancer. It was produced at Roswell Park Memorial Institute (sp act $\sim10^6$ U/mg protein and stabilized in human albumin; albumin alone served as a control preparation).

About one-half the treated nodules were markedly inhibited in their growth, several were reduced to 25% their original size within 2 weeks of treatment. Because mela-

noma control nodules grew rapidly, the differential effect amounted to an eightfold difference in tumor size in that disease. Histology revealed a heavy infiltration with lymphocytes in all treated nodules and a disappearance of melanoma cells [(*306*); Horoszewicz J: Personal communication].

One patient, treated daily for Hodgkin's disease with $8–10\times10^6$ U human fibroblast IF given systemically for an undetermined period had a disappearance of bone infiltration (Emödi G: Personal communication).

Leukocyte interferon

Various investigators at a number of clinical centers are presently testing HuLeIF against several types of human tumors through systemic and topical administration. The material used was from different sources but prepared according to Cantell's method and therefore comparable in titer and specific activity.

Topical applications

Ikic et al. (*307*) have used a daily, local application of freeze-dried crude HuLeIF for 2 to 3 months in patients with cervical cancer and have reported favorable effects. The same group applied crude HuLeIF in a powder form on condyloma acuminata of the uterine cervix and vagina and reported good clinical effects (*307*).

Habif of Columbia University, New York City, administered $1–3.25\times10^6$ U of HuLeIF daily intralesionally to 6 patients with metastatic mammary carcinoma. In 3, injected tumors regressed more than 50%. In 2, lesions disappeared grossly and histologically. One woman who had responded was also treated with HuLeIF given im. All her cutaneous lesions regressed by 50–70% (Habif D: Personal communication).

Systemic administration

Osteogenic sarcoma.—This highly malignant tumor of the bone usually occurs in a person who is between 10 and 25 years of age. Primary treatment is surgical, usually through disarticulation or amputation. In a historical series (*308*), fatal pulmonary metastases predictably develop in most patients within 12 months following the diagnosis and removal of the primary lesion. A small but variable proportion of patients, however, never develop metastases and are thus cured by surgery alone. The proportion of such long-term survivors (17% in historical groups) seems to have increased to 25–30% or even more in recent studies (Mayo Clinic study).

Although wide use of the historical series as control groups for trials of potential chemotherapeutic drugs has been made in the past by others (*309*), questions have recently been raised regarding their value for any contemporary trial, inasmuch as present-day prognosis of osteogenic sarcoma may have decreased in severity. For this reason, Strander and his colleagues developed a series composed of local contemporary controls, i.e., Swedish cases of classical osteogenic sarcoma, who were diagnosed by the same Swedish and United States pathologists and who received no other than primary surgical treatment in Swedish hospitals other than the Karolinska Hospital. Many possible prognostic factors have been analyzed in both IF-treated patients and contemporary controls and were similarly distributed between the 2 groups; the only exception was that more bone resections followed by reconstructive surgery (as opposed to amputations or disarticulations) were done in the IF-treated group than in the controls. However, if this difference is meaningful, it should bias the results against the treated group.

TABLE 3-1.—*Incidence of metastases in 3 groups of patients*[a]

Incidence of metastases at mo:	Group		
	IF-treated, %	Contemporary control, %	Historical series, %
6	16	27	57
18	43	60	86
60	54	76	86

[a] H. Strander has acceded to our request for the patients' and controls' original clinical records, and the Biostatistics Laboratory of the Memorial Sloan-Kettering Cancer Institute will perform an analysis of the statistical significance of the above results.

Comparisons between historical (1952–72) and contemporary (1972–76) Swedish controls indicate differences in percentage mortality, which was probably accounted for by more cases with histological types with the worst prognoses (on the average larger primary tumors at the time of primary treatment) among the historical controls.

The trial started in 1972. Consecutive patients admitted to the Karolinska Hospital included those having a normal chest X-ray on admission and those presenting with a primary tumor located in the long bones or the pelvis or with a diagnosis of osteogenic sarcoma based on concurring histological diagnoses by several pathologists (all with special expertise in differential diagnosis of bone tumors, including some NCI-designated pathologists in the United States).

Presently included in the study are 38 IF-treated patients, 30 contemporary nonrandomized controls, and 35 historical controls (table 3-1, 2). Treatment with Cantell-made HuLeIF starts immediately upon diagnosis, before surgery, with 3×10^6 U im/day for 1 month. It is then continued at the same dose given three times weekly for 17 months, at which time it is stopped. No other treatment is given. Chest X-rays are performed after intervals of 2

TABLE 3-2.—*Survival in 3 groups of patients*[a]

Survival, mo	Group					
	IF-treated		Contemporary control		Historical series	
	No.	Percent	No.	Percent	No.	Percent
0	38/38	100	30/30	100	35/35	100
6	35/37	95	28/30	93	23/35	80
12	27/31	87	23/30	77	17/35	49
18	24/27	89	22/30	73	10/35	29
24	20/23	87	18/30	60	6/35	17
36	12/17	71	10/29	34	"	"
48	6/13	46	8/26	31	"	"
60	5/10	50	5/21	24	"	"

[a] *See* footnote, table 3-1.

months for the treated patients and 5 months for the controls.

The average survival time between biopsy and death, in those patients of each group who developed metastases, also showed differences: In IF-treated patients it was 25 months, in contemporary controls it was 20, and in the historical controls it was 13 months (*310*).

Interpretation of these results is still difficult because of the nonprospectively randomized design of the trial that required the use of historical controls. A variable of unknown prognostic importance has also been introduced through the performance of more bone resections and reconstructions on the Karolinska Hospital patients, i.e., those treated with IF. We expect the results of this study to be reviewed and to undergo rigorous statistical analysis at the Memorial Sloan-Kettering Cancer Center.

Another study on osteogenic sarcoma in which lymphoblastoid IF was used as adjuvant therapy is being conducted presently in Austria under fully controlled conditions. However, it also includes 12 months of prophylactic chemotherapy with high-dose MTX (Bodo G: Personal communication).

In another trial, conducted by Kishida in Japan, 3 patients with osteogenic sarcoma metastatic to the lungs received high doses of leukocyte IF ($\sim 10^7$ U/day); 2 of the 3 showed temporary but significant regressions of pulmonary metastases (Kishida T: Personal communication).

Laryngeal papilloma.—Strander is also studying a group of 7 children with recurrent laryngeal papillomas. Histologically, virus-like inclusions can be seen in the laryngeal epithelium of patients with this condition. All treated patients had a long history of repeated surgery; a tracheostomy had to be performed on each of 2 children because papillomas had filled their larynges. Endoscopy of the larynx and biopsies allow one to follow the progression or regression of these recurrent benign tumors easily.

Treatment is with 3×10^6 U Cantell's HuLeIF (P-IF) im either once, twice, or thrice weekly. No other treatment is given.

In the first 2 patients treated, observed for over 3 years, Strander et al. noticed that following 3 weekly administrations of IF, the papillomas regressed, only to come back when treatment was stopped. In enlarging this trial, their purpose was to determine the best maintenance regimen, i.e., that which could maintain patients tumor free on a biweekly schedule. One child, maintained for a while on a once-a-week regimen, is now untreated and still tumor free.

Regressions induced by IF in all 7 patients occurred gradually over several months and were complete. Relapses were noticed within 2 months of cessation of treatment. Regression and relapse have been followed several times on each patient, so there is no doubt as to a cause-and-effect relationship between HuLeIF treatment and the regression of these laryngeal papillomas (Strander H: Personal communication).

Bladder papilloma.—A small number of cancer patients with a variety of tumors (1 to 3 patients/tumor type) was recently treated in Denmark with locally produced leukocyte IF. Most results were negative or inconclusive (*311*).

However, 3 patients, who, for periods from 2 to 7 years, had multiple grade II and III bladder papillomas exhibited regression and disappearance of papillomas after 2 to 17 months on 4×10^6 U of IF three times/week. In one of these, they had to change the treatment regimen from three times weekly to daily to obtain regression. These patients are presently papilloma free and untreated. However, follow-up time without treatment is still short (Osther K: Personal communication).

Multiple myeloma.—Strander's group (*312*) reported significant antitumor activity by HuLeIF in 4 patients with multiple myeloma who were treated with 3×10^6 U daily im for 3 to 19 months. A complete remission was documented in 1 patient with Bence-Jones myeloma in whom urinary light-chain excretion decreased from 13 to 4 g/24 hours and in a patient with IgA myeloma in whom the IgA level decreased from 1.8 to 0.12 g/100 ml; no protein component could be detected in the serum. Two other patients with IgA and IgG myeloma exhibited a significant partial response.

Sixteen other patients have apparently been treated in the same way but have not been reported. Apparently, of 12 patients given IF with no prior therapy, 1 died early, 3 exhibited complete remissions, 4 had partial remissions, and 4 had no response. Among 4 patients who were already demonstrably resistant to chemotherapy, 1 patient exhibited a partial response, and the others are still being evaluated in the early stages. These results obviously are most encouraging, though they cannot be evaluated because of insufficient information.

Since February 1978, doctors at M. D. Anderson Hospital and Tumor Institute (Gutterman JU, Blumenschein GR, Alexanian R: Personal communication) treated 10 patients with myeloma mostly with 3×10^6 U im daily for 8 weeks. The details of the study showed the following: Three of 10 achieved a definite objective antitumor response defined as 50% or greater decrease in serum paraprotein levels or disappearance of Bence-Jones protein excretion, or both. Three others showed improvement, which is defined as a decrease in the serum paraprotein level by at least 25% and a decrease in Bence-Jones excretion by at least 50%.

Hodgkin's lymphoma.—One patient with Hodgkin's disease with lymphocytic predominance in stage IVB was treated in Sweden. He had temporary but considerable improvement while on IF therapy, with decreased lymph nodes and pulmonary infiltrations, and normalized laboratory values (*313*).

Of 3 other patients, 1 had a complete remission and 2 had partial of 1–6 months duration following treatment with $3\text{–}10\times10^6$ U of HuLeIF (Emödi G: Personal communication).

Non-Hodgkin's lymphomas.—Merigan (*314*) treated 6 patients with B-cell lymphomas on an ambulatory basis with a 30-day course of 10^7 U Cantell's P-IF/day im in 2 doses. The average blood IF levels achieved were 229 ± 35 U/ml. All developed a depression in granulocyte counts almost immediately after starting on the treatment. However, counts returned to normal upon cessation of this therapy. Three patients had rapidly advancing diffuse histiocytic lymphoma and none responded.

The 3 other patients had indolent, nodular lymphocytic, poorly differentiated lymphoma. Their disease had never

been treated before; it was easily measurable because it was advancing and they had abnormal retroperitoneal and peripheral lymph nodes demonstrated on lymphangiography. These 3 patients had parallel responses, i.e., dramatic resolution of abnormal lymph node masses starting 2 weeks after treatment was initiated. After therapy was stopped, resolution continued up to 6 months for 1 patient, and 1 year and 6 months for the 2 others.

As an extension of these studies, Merigan is attempting to give other patients pulse treatment for shorter periods, i.e., 1-week series instead of 1 month.

Gutterman also treated lymphoma patients as described above for his myeloma patients. Six had the nodular form of poorly differentiated lymphocytic lymphoma, 2 of whom had complete remissions, 1 had a partial remission (i.e., >50% lymph node regression), 2 exhibited a less than 25% response, and 1 progressed under IF treatment.

Another patient with hystiocytic lymphoma did not respond to 3×10^6 U daily, but when the dose was escalated to 9×10^6 U daily, a large cervical tumor mass disappeared, although an abdominal mass did not.

Leukemias.—Gutterman also treated 4 patients with CLL, 2 of whom failed to respond, 1 had a partial remission while she received IF for breast cancer, and 1 had a 25–50% improvement in nodes and spleen for 5 months.

Merigan is following a patient with CLL who had a lymphocyte count of 120,000/ml at the start. Cell numbers declined with a week's course at 10^7 IU/day but went up at 2.5×10^6 IU/day and fell again when the dosage was increased (Merigan TC: Personal communication).

Patients with acute leukemias were treated with IF by Hill and associates of the Wadley Institute in Dallas, Texas (*315*) in trials begun in 1977. Leukocyte IF was made locally following Cantell's method. Hill decided from the start that higher IF dosages than had previously been used in this disease and that had resulted in no or inconclusive results would be attempted (*33*). In the early study, dosage was 10^5 U/kg body wt, capable of inducing about 60 U of peak IF blood levels. Hill and colleagues gave iv from 0.25 to 2×10^6 U/kg body wt. The total daily dose was divided into 4 injections given at 2-hour intervals. Treatment was continued daily for a few days to 2 months. No adverse effects were observed, even following an intrathecal administration of 10^6 U to an 8-year old. Only chills, sometimes with fever, were occasionally noted at the beginning of therapy.

Five patients were treated, 3 with ALL and 2 with acute granulocytic leukemia. An antileukemic effect was observed in 3 of 3 patients with ALL and 1 of 2 patients with the granulocytic type.

Metastatic breast cancer.—Little work has been done in patients with solid tumors. The most extensive studies were done by Gutterman and co-workers on 17 patients with breast cancer [(*316*); Merigan TC: Personal communication]. Patients were selected who had superficial metastases, which allowed objective measurements of tumor size. Most of the patients had been previously heavily treated, and many had failed to respond to conventional therapy. Two doses were used, either 3×10^6 or 9×10^6 U daily. Of 11 women given the low dose of IF, 4 exhibited a partial response and 1 a less than partial response; of 6 women given the high dose, 2 exhibited a partial response.

Gutterman et al. made the following general observations: Breast cancer patients tended to have higher circulating IF levels than did those with lymphoma and myeloma. They speculated that this may be due to more binding of IF to WBC in the latter patients. Treatment with IF had a clear-cut effect on WBC counts: a median count of 2,600 leukocytes, with depression of granulocytes and lymphocytes. No suppression of DTH was noted, but the response of lymphocytes to mitogens was transiently decreased. Despite this, no viral infection has been observed in the series of 38 patients, many of whom were treated for over 1 year.

Side effects have decreased with the use of more purified material, i.e., P-IF with sp act over 10^6 U/mg protein. Fever and fatique have been minimal; they are clearly dose and age related. Minimal loss of weight and hair occurred in some patients.

Conclusions

Therapeutic antiviral effects of IF have been demonstrated in animals and man. Therapeutic, and even perhaps curative, antitumor effects were reported in animals under certain regimens of treatment with IF and/or treatment combinations. Antitumor effects in man, including tumor regressions, were observed in uncontrolled studies with IF alone at doses which elicited little or no toxicity.

The number of cancer patients studied thus far is small, and neither the degree nor the duration of possible therapeutic benefit to patients can be evaluated. However, neither maximum dosage nor optimum regimen of treatment has been achieved. Room for improvement is considerable in the quality of the preparations used for clinical therapy such as through more extensive purification. Experimental therapy with IF in viral and neoplastic diseases in animal model systems and clinically is impeded by the scarcity of preparations, which remains a major obstacle to such studies.

POSSIBLE TOPICS FOR A RESEARCH PROGRAM IN THE INTERFERON FIELD

INTRODUCTION

New knowledge needs to be acquired so that IF prophylaxis and therapy can be brought to rational and effective clinical application. Some of the questions outstanding in the field and some of the developmental problems that need to be overcome are outlined below.

INTERFERON INDUCERS

Several diverse natural and synthetic substances are potential IF inducers. Those inducing Type I IF have been classified into: 1) those capable of stimulating at least 10^3 U/ml of acid-stable IF in either cultured cells or the blood stream of animals when given in microgram amounts; 2) those often inactive in cultured cells, inferior both in amounts of stimulated IF and the milligram quantities of

inducer needed per kilogram body weight. This group encompasses antigens and mitogens which induce a special Type II IF.

Effective, noninfectious, and nontoxic IF inducers would have clinical value because endogenous IF could complement the administration of the exogenous type and each might be useful as a therapeutic agent alone.

GENETICS, INDUCTION, AND REGULATION OF TRANSCRIPTION

The events immediately following the interaction between an inducer and the cell surface membrane are not known; whether internalization of the inducer or of a putative inducer–receptor complex occurs is unknown, as is the mechanism of genetic activation itself. Mouse and human cells appear to carry several structural genes for IF; in man–mouse cell hybrids, each of 3 human chromosomes (2, 5, and 9) have been found necessary and sufficient alone for human fibroblast IF production. In addition, diploid human fibroblasts normally make some leukocyte IF, and leukocytes make some fibroblast IF, both in small proportions. Because fibroblast and leukocyte IF are, for the time being, believed to be products of different genes (on the basis of the faithful translation of their mRNA in heterologous systems according to cellular origin), one possible interpretation of the above is that human cells have multiple structural genes for a single type of IF located on different chromosomes, in addition to at least 1 gene for another type. Another explanation could be, however, that different posttranscriptional modifications of a single primary transcript occur in different types of cells, i.e., they are determined by epigenetic factors. Further research must establish which is correct.

Virtually all nucleated cells respond to IF induction. An inducing stimulus is required to initiate IF gene transcription because IF mRNA is found only in induced cells; its production is de novo, and because it has been translated in heterologous and cell-free systems, we have direct evidence for its existence. However, IF mRNA activity is short-lived; it decreases and becomes undetectable within a few hours of induction. Therefore, cells must have a regulatory mechanism(s) controlling IF production at the *transcriptional* level; inasmuch as the normal repressed state appears due to active repression, induction must bring about a derepression. The nature of the repressor(s) and the precise location on the genetic map of the structural and regulatory genes for the repressor are unknown.

Several theories regarding derepression mechanisms are being tested; several mechanisms may be involved because the kinetics of IF production vary in the same cells or in the same animals with different inducers. Certain IF structural genes may be specifically activated by certain inducers, as the physicochemical properties of the IF produced also vary with the inducer used. However, different post-transcriptional modifications that could be directed by epigenetic factors cannot be excluded at this time. When molecular probes (mRNA and cDNA) for IF become available, they will allow studies on this system at a fine structural level, and allow, in particular, the mechanisms which effect the turning on and turning off of gene transcription to be investigated at the molecular level. Investigations on transcriptional regulation in eukaryotic cells are important, and they have become possible in any system only recently.

Because of the de novo nature of the biosynthesis of IF and of their mRNA (sensitive biological assays exist for both), the IF system is considered one of choice for research on the control of transcription. Moreover, such studies also have a practical importance for *IF* genes because they may contribute directly to increased yields. Constitutive, high-yield IF-producer cells could be developed on the basis of better knowledge of the system's genetic map; human cells enriched for multiple copies of selected structural genes, with deletions of regulatory genes, could be engineered through somatic cell genetic techniques. Such methods would reduce the cost of IF production in eukaryotic cell culture systems.

INTERFERON PRODUCTION AND ITS REGULATION

As in other studies on mammalian protein biosynthesis, IF mRNA translation and its control have been studied with the use of inhibitors in DNA-dependent RNA synthesis to uncouple transcription from translation. Considerable but still incomplete information has been gathered on the sequence of intracellular events leading to the production of extracellular IF. Nuclear IF mRNA is polyadenylated and found associated with membrane-bound polysomes in the cytoplasm where translation occurs; we have information on the site of IF glycosylation. Its secretion is an energy-requiring step and is impeded by disruption of microtubule formation. Thus the biosynthesis of IF seems to follow a general pattern characteristic of other secretory proteins.

The concurrent treatment at specific times of induced cells with RNA and protein synthesis inhibitors revealed an apparently paradoxical phenomenon, i.e., that the rate and duration of IF synthesis can be *increased* considerably, resulting in yields up to 100 times higher than they are in cells which are only induced. This phenomenon has been called "superinduction" and has been put to practical use for large-scale production from fibroblast-type cells. However, due to the irreversible nature of the inhibition of RNA synthesis by dactinomycin, routinely used superinduced cells must be discarded after a single superinduction. It has recently been shown that experimental use of the reversible inhibitor of RNA synthesis 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole[3] allows the same cells to be reinduced several times, and, therefore, a significant reduction in the cost of human fibroblast IF production is achieved, Unfortunately, this has not been available in sufficient quantity from any source for large-scale production.

Studies on the mechanisms of superinduction have allowed postulation of the existence of a regulatory protein(s) involved in the cessation of IF biosynthesis shortly after its induction. The IF system appears to be

[3] The 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole is commercially available from Calbiochem, San Diego, California.

under the control of a repressor, which may operate at two levels, i.e., to shut off transcription and to inactivate the IF mRNA because 1) the latter substance disappears rapidly following the activation of this control mechanism, and 2) it is stabilized by the use of dactinomycin after *IF* gene transcription is complete. Because it remains active in various cell systems for about as long as hyporesponsiveness lasts, the repressor may also be responsible for the phenomenon of hyporesponsiveness to repeated induction.

In addition, IF can be involved in the regulation of their production through their ability to "prime" and "block" synthesis in cells treated, respectively, with low and high concentrations of IF before induction. Priming has been demonstrated with highly purified IF, whereas blocking has not, which may indicate that another factor, co-induced with it and present in partially purified preparations, is the blocking agent. Blocking requires RNA and protein synthesis for expression, but priming does not; priming could result from a cell surface membrane effect. Neither mechanism is understood, and, for the time being, the nature of the putative blocking factor is totally unknown.

Further studies on superinduction are essential, inasmuch as they deal with mechanisms which control protein biosynthesis at the transcriptional and the translational levels in eukaryotes. From the practical standpoint, their importance lies in further possible increases in IF yields from fibroblast-type cells. They could perhaps also result in the development of superinduction procedures applicable to lymphoid cells, which would decrease the cost of production of leukocyte IF.

INTERFERON MESSENGER RNA

Many studies were undertaken as soon as it became obvious that IF mRNA could be translated faithfully in heterologous systems and that advantage could be taken of their species specificity, including the unique antigenicity of the translation products; indeed, all of this allows the IF mRNA of different animal species to be distinguished and even those for different types of human IF to be identified with the use of various neutralizing antisera on their translation products. Much work is now ongoing with mRNA from the mouse and the human species, including the human fibroblast, leukocyte, and Namalva (lymphoblastoid) IF.

Translation of IF mRNA can occur in heterologous cultured mammalian cells, by which they are taken up and from which the translation product is secreted. It can also take place in *Xenopus laevis* oocytes into which mRNA must be injected and from which translation products must be extracted, or in various cell-free, protein-synthesizing systems, including the wheat germ system. Oocytes can even translate nonpolyadenylated messages (IF mRNA) from which the poly A tail has been removed enzymatically.

In biological assays of translation products as IF, some heterologous translation systems are highly sensitive, particularly the oocyte system, which allows the detection of 2 mmol of IF mRNA, which corresponds to 10–100 IU of IF activity (or $<10^{-7}$ mg of IF protein). It has a sensitivity greater than that of any immune assay.

Recent work with IF mRNA has, first of all, shown that none is detectable in noninduced cells or following the cutoff of IF production, thus establishing that it and its mRNA biosynthesis result from a true induction process. Thus priming results in an earlier appearance and an earlier disappearance of the IF mRNA, with no increase in transcriptional efficiency, but an increase in translational efficiency, which clarifies the reason for increased IF production in primed cells. Furthermore, not only is some additional IF mRNA made during superinduction, but what there is has a markedly prolonged half-life, i.e., it is stabilized. This finding supports the assumption that superinduction inhibits the synthesis of a regulator which either blocks or degrades the IF message. Whether this effect of superinduction is specific for the IF message or affects other cellular messages also is still being debated.

On the basis of studies which have included those on sedimentation rate, target size mapping with UV radiation, and others, it can now be estimated that the message for the smaller molecular species of human IF is 850–900 nucleotides long and has a poly A tail more than 100 nucleotides long. On the basis of molecular weight determinations on deglycosylated human IF (indicating a mol wt of 15,000–16,000), the coding region of the message should contain 450–500 bases. Because IF are secretory glycoproteins synthesized on RER 50–60, nucleotides can be assigned to the N-terminal signal sequence, leaving 200 nucleotides for the noncoding region. Whether capping occurs is not known, but it is being investigated. A primary transcript larger than the mature message (from which the latter would be formed by the splicing of the coding sequences) has not been established but is considered likely, particularly because this is true for many cellular and viral mRNA. On the basis of target size mapping, the IF nuclear precursor mRNA has been estimated to be about 10,000 nucleotides long.

The half-life of the IF mRNA is short, among the shortest known for mammalian mRNA, i.e., between 30 minutes and several hours, depending on whether the cells are, respectively, induced or superinduced.

Because of the small amount present in any induced cell, estimated to be at most 0.1% of the total cytoplasmic mRNA, the complete purification of any IF mRNA has not been reported. A monoclonal antibody has not been extracted from hybridomas against any IF, nor has a sufficiently specific, high-titer antiserum IF mRNA been extracted as a single polysome-bound mRNA species. This would constitute the most efficient method for its extraction and purification, and intense efforts are presently under way to achieve it.

The availability of pure IF mRNA will allow an oligo dT-primed, reverse transcriptase-catalyzed production of IF-complementary DNA (dDNA) for recombinant DNA work and would also facilitate attempts at mass production of IF.

For the immediate future, the synthesis of cDNA for IF will allow the use of recombinant DNA technology for the solution to the industrial-scale production of IF.

ASSAYS FOR INTERFERONS

All IF assays measure biological activity, i.e., a modification of a cell function caused by IF action. Therefore, they all require live cells, and those that measure antiviral activity also require a live challenge virus. Results are recorded by the plotting of dilutions of an IF preparation against a percent of the value obtained with control cells. Such plots result in sigmoid-shaped dose–response curves, with an approximately straight-line relationship between 25 and 75% inhibition of cell function; this is the only zone within which a direct dose–response relationship is found. The variabilities inherent in all current IF assays have made necessary the establishment of human and mouse Type I international reference preparations against which any IF preparation from these species can be calibrated. Each assay includes a laboratory standard, itself previously calibrated against a sample of an international standard; thus results can be expressed as standard international reference units (IU).

The assays most commonly used measure the antiviral state induced by IF and with easily quantifiable parameters of virus replication. Among these are: inhibition of either plaque formation or virus-induced cytopathology caused by lytic viruses (in the latter case either through direct reading of cytopathogenic effect or the colorimetric measurement of vital dye released by cells); reduction of single-cycle virus yields as measured by infectivity, hemagglutination, hemadsorption, or neuraminidase activity, depending on the virus used. The incorporation of [^{3}H]uridine into viral mRNA has also been used with cells treated with dactinomycin for inhibition of cellular mRNA transcription.

Other assays could be devised which would use measurement of other IF activities such as priming, enhancement of cytotoxicity caused by dsRNA, inhibition of cell multiplication, etc. These cell functions are less easily and rapidly quantified than the antiviral effect and therefore not routinely used; on most cells, the ratio of antiviral activity over any of them appears to be constant. However, this is not so for all cells (*see* section on Cell Growth Inhibitory Effect), and assays for other than the antiviral activity of IF should be developed.

In particular, it would be most important for investigators to develop an in vitro assay that would correlate directly with the *antitumor* activity of IF, which can be measured only in vivo after long-term, high-dose administration. That all types of IF, particularly the human, have the same ratio of antiviral and antitumor activity is not established. It seems, in fact, that the Type I and Type II mouse IF vary greatly in this respect (although it remains to be demonstrated, through its complete purification, that the high antitumor activity of mouse Type II per unit of antiviral activity resides in the moleeules with antiviral activity).

The development of an in vitro antitumor assay for mouse IF must either await knowledge of the precise mechanisms of its antitumor action, or, at the very least, that *quantitative correlations* be established empirically between antitumor activity in the mouse and an IF effect in vitro, e.g., enhancement of macrophage or NK cell cytotoxicity for tumor cells.

The simplest and the most rapid antiviral IF assay presently used involves the direct reading under the microscope of inhibition of cytopathogenic effect in cells grown in 96-well Microtest plates, with vesicular stomatitis virus used as a challenge virus. End points are read as 50% protection (2+) in plates in which cell controls show intact cell monolayers (4+) and virus controls 100% of this effect (−). Readings can reliably be done with 0.5 $\log_{10}$ dilutions and can be interpolated to 0.3 $\log_{10}$ differences. This assay is the most convenient when large numbers of samples must be tested because of its relative speed (total time needed is about 36 hr) and relative economy of materials. For added sensitivity in assays of human IF, GM258 cells (trisomic for chromosome 21) can be used. For more accuracy and objectivity, particularly when *low* concentrations of IF are measured, inhibition of virus yield is preferred.

Because the biological activity of pure IF approximates $1\text{–}3\times10^9$ U/mg protein, the above IF antiviral assays, which easily detect 1 IU or less, measure the activity of picogram quantities of IF protein, i.e., $10^6\text{–}10^7$ μg protein.

Methods have also been developed for the detection of single IF-producing cells. A procedure similar to that used for an "infectious center" assay is used, i.e., cells induced for production are plated over a monolayer of virus-infected cells, and the culture is overlaid with a semisolid agar-containing medium. The IF-producing cells are revealed by discrete foci of intact monolayer, whereas the surrounding background is destroyed by the virus-induced cytopathogenic effect. A count of such foci can reveal the proportion of IF-releasing cells in a population of induced cells, and the size of the foci allows the relative amount of IF produced by each cell to be estimated.

Although biological assays of this type have the advantages of high sensitivity, they also have obvious disadvantages. An RIA would be particularly useful. Investigators have tried to produce monoclonal antibodies to IF, as well as radioactive IF. The latter product has been attempted with human fibroblast IF and direct labeling with iodine-125 and thus far has resulted in a product with biological activity, stable for 1 month, and a specific radioactivity of 2–4 μCi/μg protein. The availability of either radiolabeled specific antibodies or pure IF would allow an RIA to be developed which would have many experimental and clinical uses. For example, it would allow us to determine the number of IF receptors on cells, whether different kinds of receptors exist for different IF species, or whether IF–receptor complexes must become internalized to establish the IF phenotype, etc. An RIA would also be useful for determining levels of circulating IF in the blood and its fate in vivo, i.e., whether it binds preferentially to certain tissues or not.

PURIFICATION OF INTERFERONS

Our goal in IF purification is twofold: 1) to obtain a product with measurable activity in vivo that can conveniently and safely be administered clinically in a small volume; 2) to result in "pure" IF, so that their molecules

can be completely characterized physically, chemically, and biologically, and for their in vivo properties.

The first goal has nearly been reached through conventional approaches to protein purification, except for needed improvements in recovery of total activity (now still often <50%) and additional simplifications.

Although investigators are intensively pursuing the second goal, and it may soon be reached, much remains for them to accomplish, e.g., 1) much improvement in recovery of activity (now only 1–5%), 2) a general reduction in the number of steps required, 3) a reduction in the cost of the equipment and the reagents needed, and 4) the synthesis according to preparative scale.

Longstanding efforts toward complete purification have demonstrated that the native Type I IF from a single animal species is a family of glycoproteins, highly heterogeneous at least for their size and charge. Although all have antiviral activity (by definition), subtle differences may occur in cross-species activities, tissue-binding specificities, and/or ratios of antiviral to non-antiviral activities between major types of IF of the same species and even between minor molecular species within one type. These differences are presently being investigated on isolated molecular species.

One advance achieved in the early 1970's has been the observation that IF inactivated by boiling in SDS can regain biological activity because their primary structure is not destroyed by this treatment. This has allowed the application of dissociating conditions to these proteins so that the aggregates are broken up and conformational homogeneity is obtained. The use of SDS–PAGE could then allow real molecular weight differences to be detected between the molecular constituents of various preparations. The SDS–PAGE and the assay of IF activities in eluates of polyacrylamide gel slices is the method now routinely used in purification.

Both physicochemical and immunological (affinity chromatography) techniques were applied to their purification, often in combination. Different schemes or sequences of various steps had to be developed for IF from different species and even for human fibroblast IF and HuLeIF that attest to the distinct molecular properties of the leukocyte type.

Molecular species of leukocyte, fibroblast, and lymphoblastoid IF and mouse IF have now been purified to size and charge homogeneity, although in extremely minute amounts (Type II IF have only been partially purified because of the present lack of large starting amounts). All the purified types exhibit exceedingly high specific antiviral activity, i.e., $1\text{–}3\times10^9$ IU/mg protein, as well as a number of other biological activities characteristic of partially purified IF preparations, including cell growth inhibitory activity in vitro and, for the mouse IF, also immunomodulatory and tumor inhibitory activities in vivo.

INTERFEROIDS

According to recent work, chemical cleavage under mild oxidative conditions converts heterogeneous molecular populations of HuLeIF into a single narrow band in SDS–PAGE that also focuses into a single narrow band in focusing gels, and, therefore, is homogeneous for size and charge. It has been determined to be completely or largely deglycosylated IF and to be fully active in cultured cells.

Subsequently, it has been demonstrated that cells, including human leukocytes, can synthesize carbohydrate-free IF in the presence of inhibitors of glycosylation and that this synthesis can occur without any reduction in its activity produced per cell. Deglycosylation was always accompanied by a broadening in cross-species activity.

Deglycosylated IF, called "interferoids," have hydrophobic properties similar to those of native IF and can be purified by the same procedures.

Conversion of IF to interferoids by treatment with acid periodate has been introduced as a step in the purification procedures applied to leukocyte IF and has allowed pure IF protein to be isolated as discrete spots in two-dimensional O'Farrell-type gels. This protein is being eluted and used for the determination of its amino acid composition and sequence. Similar determinations have been attempted by several investigators on single bands with IF activity, isolated from one-dimensional gels, obtained with nondeglycosylated human fibroblast and lymphoblastoid IF or with mouse IF.

Although the true purity of the single-band isolates remains to be determined, isolates from two-dimensional gels fulfill much more stringent criteria of purity and should provide choice materials for amino acid sequencing studies.

However, a key question regarding the activity of interferoids in vivo has not been answered. We must determine how their toxicological and pharmacological properties compare with those of native IF because the lack of carbohydrate may modify their stability in vivo, their clearance rate, or other properties. This crucial information must be obtained in animals first and then in humans because future chemical synthesis or prokaryotes that express a human *IF* gene will be producing naked polypeptides. Should these polypeptides be shown to require modifications for in vivo activity, such as the attachment of sugar moieties, the task of producing active material on an industrial scale would be more difficult and costly.

The demonstration that IF activities reside entirely in the polypeptide part of the native molecule has important theoretical and practical implications: 1) It points to structural and perhaps functional analogies between IF and protein hormones; 2) it allows purification procedures to be performed on homogeneous material and the acquisition of a pure product according to stringent criteria; and 3) it offers the hope that either chemical synthesis or prokaryotes will produce materials that may be clinically useful without further chemical modifications.

FRAGMENTATION OF INTERFERONS

Given the likelihood that IF share a small active core, perhaps even one that is fully active across species barriers, researchers are attempting to cleave the IF polypeptide chemically or enzymatically to isolate such a core. This work, in its early stages, is most important because both the use of recombinant DNA technology and that of chemical synthesis for the production of material with IF activity will present difficulties directly proportional to the

size of the polypeptide needed. The active fragments which may result from this research must be assayed for their range of activities, not only in vitro but in vivo including across species barriers, and tested for their toxicological, pharmacological, and antigenic characteristics. The isolation of active fragments or of a true active core common to all IF has theoretical and practical importance.

PROSPECTS FOR INTERFERON PRODUCTION WITH RECOMBINANT DNA TECHNIQUES

Tested recombinant DNA technology could immediately be applied to the production of human IF by prokaryotes. Indeed, the human somatostatin and insulin genes have now successfully been inserted in, transcribed, and translated by bacteria to yield a biologically active product. Necessary prerequisites to such work are either a cDNA for human IF, or knowledge of at least part of the sequence of the active polypeptide, preferably both. Investigators can attempt obtaining a cDNA for human IF by a direct approach, i.e., by extraction and purification of IF mRNA from induced human cells, which has proved difficult due to the low content of the messenger type in human cells. For this reason, this approach has not been fruitful.

An alternate approach presently being attempted is an indirect one; it involves first the synthesis of a cDNA to mouse IF mRNA, and then its use as a probe for extraction of the human gene for cloning. The indirect approach is made possible by the existence of a mouse cell line which, when induced with UV-irradiated reovirus, is capable of producing 100 to 1,000 times more IF per cell (and therefore also proportionately more IF mRNA) than any known human cell. Using these mouse cells, one can isolate and purify mouse IF mRNA and a full-length cDNA. A full-length cDNA has been synthesized and was purified by several cycles of hybridization to the purified polyadenylated mouse mRNA extracted from induced and uninduced cells, until a fraction was obtained that hybridized only to the poly A-containing mRNA from induced cells. This fraction, which, in all likelihood, is pure IF cDNA has then been made double stranded, tailed, and inserted into plasmid pBR322. Transformants were isolated. On the basis of past results with somatostatin and insulin, some investigators believe that the mouse *IF* gene will be expressed fully and hope that its biological activity will be detectable. This expression would allow its cloning, i.e., the selection and outgrowth of a genetically homogeneous population of those bacterial cells expressing only the mouse *IF* gene. The gene itself would then be extracted in particles of the recombinant bacterial DNA with purified mouse IF mRNA which will anneal only to it. Once so extracted in a pure state and made single stranded, it can in turn be used for hybridization to the human *IF* gene with which it may have sufficient base sequence homology to bind efficiently. Once isolated, the *IF* gene will be inserted into plasmid DNA and cloned as was done for the mouse gene.

Knowledge of the human IF amino acid sequence would be most helpful to this work because it would allow us to make correlations between amino acid and base sequences as isolated by the cDNA probe. Also, a pure IF available in sufficient amounts would allow the development of an RIA which would facilitate the identification of the human gene product in bacterial extracts. Alternatively, the desired DNA sequence could be identified by competition experiments, i.e., binding the sequence to IF mRNA and so reduce the activity of this mRNA, but this would be much more laborious.

Another avenue toward the production of human IF by bacteria is offered by knowledge of its amino acid sequence alone; knowledge of the sequence allows derivation of a base sequence coding for it and synthetic construction of a DNA probe. This approach has been used successfully for somatostatin. Codons can actually be selected that are preferentially read by bacterial cells. If necessary, and provided that its complete amino acid sequence is known, a DNA sequence can be constructed that codes for the whole IF polypeptide. (Such a task, however, would require about 1 yr for completion.) Thus instead of the natural human gene, an artificial but fully functional one would be used for insertion and expression in bacteria.

It has been estimated that, as a result of appropriate genetic manipulations, bacteria could be made capable of producing 10^5–10^6 molecules/cell of the product coded for by an inserted DNA sequence. This last approach has the advantage of bypassing the need for purification of either the human or the mouse IF mRNA in the production of cDNA probes enzymatically. Given the likelihood that one or more at least partial amino acid sequences of human IF will soon be known, this approach may also provide an early solution to the cloning of a DNA sequence coding for human IF.

Industrial production capability resulting from any successful recombinant DNA manipulation would have virtually unlimited capability and be most cost efficient.

ANTIBODIES TO INTERFERONS

The IF are effectively antigenic in heterologous species, and excellent titer antisera ($\geq 10^6$ U of neutralizing activity/ml) have been obtained in several species against human and mouse types. However, none of the existing antisera is of sufficient specificity for either their complete purification by affinity chromatography, the isolation of polysome-bound IF mRNA, or the development of RIA. All the existing antisera have been prepared against preparations which, though of high titer ($\geq 10^6$–10^7 U/ml), represented an antigenic mass of which only 0.1–1% was IF protein.

Investigators have tried to produce monoclonal antibodies through the standard hybridoma technique (i.e., fusion of spleen plasma cells from mice immunized with an IF preparation to mouse myeloma cells, which results in monoclonal antibody-producing hybrid cells with an unlimited ability to propagate in cell culture). These attempts have allegedly been unsuccessful, perhaps because detection of antibody to any IF must now be done through an *activity neutralizing* assay. Any single monoclonal antibody may have too narrow a specificity to be neutralizing by itself. However, other possible approaches to the resolution of this problem exist, and it is important that they be tried and that a library of monoclonal antibodies to the antigenic determinants of the human IF polypeptide(s) be established.

Techniques would be invaluable that would allow us specifically to identify IF protein present in small amounts (such as for screening for *IF* gene expression in bacterial transformants) or to bind IF protein and thus efficiently extract IF molecules from crude preparations with column-bound antibodies. The availability of monoclonal antibody(ies) could be crucial to simplified and complete purification of IF on a preparative scale and also to various areas of research.

POSSIBLE TISSUE SPECIFICITY OF INTERFERONS

Thus far all types of IF from a number of vertebrate species are capable of inducing an antiviral state in all cells sensitive to their action and apparently also inhibiting cell multiplication. However, sensitivity to their growth inhibitory effect not only varies markedly from cell type to cell type but on the part of a given type of cell in response to the 2 major types of human IF. This last observation raised the question of whether IF types, or even particular molecular species within 1 type, may have tissue or organ specificity with regard to growth inhibition and perhaps also other IF activities.

Inasmuch as an ability to inhibit cell multiplication may be an important component of the antitumor effect of IF, clarification of whether different human types and their molecular species have particular tissue affinities is important; if so, these should be quantitatively evaluated for their kind and range, in various cultured cells and in nude mice carrying human tumor cells. The results of such studies would allow the rational and economic use of whatever type of human IF is available for clinical trials.

INTERFERON-CELL INTERACTIONS

Required for all IF activity are cells that are capable of synthesizing RNA and protein and carrying a postulated, specific, cell surface receptor for which genetic determinants are located, e.g., in human cells in chromosome 21. The chemical and structural bases for this affinity or the receptor's nature are not known; nor is it known why IF have strong affinity for homologous cell receptors and also, sometimes, for those of phylogenically distant species, nor whether species specificity does in fact reside at the level of the receptor.

Marked changes in cell surface properties occur following IF binding to cells. Their meaning and especially their relationship to subsequent intracellular changes, both structural and biochemical, are not understood. Whether IF molecules, a large proportion of which elute spontaneously after binding, just signal cells through a brief interaction or whether at least some become internalized either alone or complexed with their receptor is unknown. However, we do know that IF must first interact with the cell surface membrane to be active. If antibody to IF is present in the medium surrounding cells induced for its production, the cells do not develop an antiviral state.

No one has been able to produce enough radiolabeled IF or highly specific labeled antibodies to IF species that would allow precise quantitative studies of small amounts of IF protein and the tracing of its possible intracellular fate. It is appealing for one to speculate that IF have an intranuclear role because of their strong affinities for polynucleotides, which are being exploited in purification schemes. Studies in these areas have relevance to the understanding of the mechanism(s) of gene activation brought about by IF and perhaps to the control of eukaryotic gene transcription in general.

To our knowledge, no extensive attempts have been made at isolating and characterizing the putative specific receptors for IF. Basic research on their nature, the fate of the IF–receptor complex, and the events which lead to a modulation of genetic expression in IF-treated cells have fundamental importance.

CELL GROWTH INHIBITORY EFFECT

The inhibited growth of normal and transformed cells in the presence of IF occurs in vitro and in vivo, such as on regenerating liver cells and transplanted tumor cells. Whether one or the other kind of cell is more affected is neither clear nor easily determined. Different types of cells vary in their sensitivity to growth inhibition by IF in a way that does not directly correlate with their sensitivity to the antiviral effect.

To obtain a growth inhibitory effect measurable as a decrease in population doubling time, one usually has to culture cells for several days in the presence of IF at a higher concentration than is needed to observe a marked antiviral effect. This is not necessary for all cells, however, as some are completely inhibited in their growth by 1–10 IU of IF/ml in the medium. Some human cells also vary in their sensitivity to different types of IF, e.g., in humans, lymphoid cells are more inhibited in their growth by leukocyte IF, whereas osteogenic sarcoma cells are more inhibited by the fibroblast type (*see* Tissue Specificities).

Few investigations on the *relative* sensitivity of cells to the growth inhibitory effect of IF have been conducted. In addition, different parameters of cell growth have been studied, such as numbers of cells in a total population, or cell-saturated density, plating efficiency, or capacity to form colonies in semisolid media. Although all these parameters are important and are apparently affected by IF treatment, they may be mediated by different mechanisms, but one cannot use them indiscriminately to draw conclusions on the effects of IF on cell growth.

Just an increase in the doubling time of a randomly cycling cell population could be brought about by our 1) blocking the cells in one or more phases of the cell cycle; 2) shunting the cells out of the cell cycle into a quiescent (G_0) state; 3) slowing the average rate at which all cells progress through the cycle, with all phases of the cycle being affected to a similar extent; 4) halting the cells at random in any stage of the cycle with or without a general slowing. Present opinion concerning these alternatives is conflicting. Most reports claim a generalized slowing down of the cycle usually obtained with tumor cells; others state that IF acts by shunting those cells (fibroblasts most frequently) into a quiescent state during the G_1 phase that normally exhibit a "restriction point" in G_1. Moreover, the question of the general reversibility of the effect is still open; although it appears reversible for most cells, this may not be so for all of them, and some may be lost to the population, particularly with prolonged treatment.

A large amount of information could be obtained rapidly in this area with the use of flow cytofluorometers coupled to computers and with methods of fluorescent staining for nucleic acids that provide precise information on the proportion of cells in a population in any phase of the cell cycle. Such studies have barely begun, and it is not known whether any of the biochemical changes observed in IF-treated cells are responsible for the inhibition of cell growth, i.e., whether the antiviral and the cell growth inhibitory effects of IF share common biochemical pathways.

Because of the possible importance slowed cell growth may have as a component of the antitumor effect in vivo, the effect of IF on the cycling behavior of different kinds of cells should be studied intensively with different IF on both normal and malignant cells in vitro and in vivo.

IMMUNOMODULATION BY INTERFERONS

In lymphoid cells, the expression of an induced phenotype includes: 1) a decrease in the rate of cell multiplication during the proliferative phase of the immune response and 2) enhancement of various specialized lymphoid cell functions, such as antibody secretion, cytotoxicity, and others, during the nonproliferative phase of the response. These two preponderant effects result in either a measurable overall inhibition or an enhancement of an immune response.

In vitro, IF can affect the primary and the secondary antibody responses to thymus-dependent (SRBC) and independent (LPS) antigens. The concentration of the IF used and the timing of its administration in relation to the antigenic challenge determine the nature and amplitude of the effect: The higher the concentration of IF and the earlier it is given during the immune response, the more profound is the immunosuppression obtained, with the maximum effect obtained when IF treatment is given just before or with the antigen. In vitro, IF can have an enhancing effect on the antibody response when sensitized cells are treated at low doses several days after challenge with the antigen. Through experiments with isolated cell populations, researchers have shown that the effect on antibody production is a direct effect on B-cells and not on T-cells or macrophages.

In vivo, similar effects have been reported in primary and secondary humoral responses in the mouse, i.e., immunosuppression resulting from early high-dose IF administration and enhancement resulting from late lower dose administration. A direct action on B-cells can again be demonstrated when maximum suppression is obtained if IF is given 4–48 hours before the antigen and a significant enhancement if given 96 hours after the antigen. The effect involved IgM, IgG (measured, respectively, by sensitivity or resistance to treatment with 2-mercaptoethanol) and also IgE (measured by heterologous, adoptive, cutaneous anaphylaxis reaction by mouse cells in the rat).

In vitro IF treatment markedly inhibits lymphoid cell proliferation, as well as synthesis of RNA and DNA, in response to mitogens (whether PHA, Con A, PWM, and LPS), which indicates that both T- and B-cells are affected. Inhibition of lymphoid cell proliferation in response to allogeneic lymphocytes is also observed in mixed lymphocyte cultures, i.e., mixed lymphocyte reaction. This effect, which occurs in vivo if IF is administered from 24 hours to 4 days before measurement of a proliferative response in vitro, has also been observed in man. In addition, IF treatment can: 1) inhibit both the afferent and efferent arcs of the DTH reaction to various antigens (the immunosuppressive effect of viral infections on DTH has been attributed to endogenous IF); 2) delay graft rejection and GVH reaction and also inhibit the proliferation of allogeneic mouse spleen cells in irradiated mice; 3) enhance the number of macrophages with phagocytic activity and the level of this activity in vitro and apparently also in vivo; 4) enhance the expression of histocompatibility antigens on mouse tumor cells, thymocytes, and splenocytes within 12–24 hours; and 5) enhance cell-mediated cytotoxicity within 3–4 hours.

This last finding has been considered as having significant potential importance to an understanding of the mechanism(s) of the antitumor effect of IF in vivo. However, over recent years, the concept of immune surveillance against neoplastic cells involving T-cells as effectors of cell-mediated antitumor responses has been modified in the light of new evidence obtained in nude, thymectomized, and irradiated mice. Furthermore, non-T-dependent, noninduced cytotoxic cells may exist as mediators of immune surveillance against tumors.

A natural H-2 and species-independent cell-mediated cytotoxicity has been described, mainly in the human and mouse, that is capable of bringing about the lysis of various virus-infected and tumor cells in vitro. It is mediated by small lymphocytes (NK), which are without adherent properties, have Fc receptors but no surface markers for T- and B-cells, and differentiate from bone marrow precursor cells.

An elevation of NK cell activity has been reported in mice given injections of *C. parvum,* tumor cells, or viruses, and in rats treated with poly I:C. All these agents are IF inducers.

On the other hand, we know that mouse IF is capable of inhibiting the growth of tumor cells in vivo, even that of the L1210*R* cells selected in vitro for *resistance* to antiviral and antiproliferative actions of mouse IF. The L1210*R* cells seem to be mutants which have lost their genetic determinants for the IF receptor because they are unable to bind IF. Therefore, they are insensitive to its *direct* effects. The in vivo susceptibility of L1210*R* cells to mouse IF antitumor effect strongly suggested that this effect must be mediated by the tumor-bearing host. Experimenters undertook to examine whether this antitumor effect could be due to an IF-induced enhancement of NK cell activity.

It has now been shown convincingly that both human and mouse IF are potent activators of natural cytotoxicity in vitro and in vivo for various target cells and that the properties of the effector cells involved are those of NK cells.

Although NK cytotoxicity can be directed against various cells irrespective of their species of origin or histocompatibility, NK cells do not kill all target cells with equal effectiveness; virus-infected and tumor cells are far more sensitive than normal cells. This has led investigators

to suspect that the target cell itself might play a role in the level of stimulation of the cytotoxic effector cell. Indeed, when incubated with PBL, various tumor cell lines are capable of inducing high levels of an antiviral activity (up to 6,000 IU/ml) which, in across-species mixtures of lymphocytes and target cells, displays an antiviral specificity for the species from which the *lymphocytes,* not the target cells, are derived. For example, this occurs in mixtures of human lymphocytes and virus-infected mouse fibroblasts in which *human* IF is produced. Some virus-infected cells are more effective in inducing the production of the antiviral factor than others, and, although contact between cells is required, the nature of the triggering stimulus is not known. It seems clear, however, that one is not dealing here with an immune-type recognition.

The antiviral factor produced has no toxicity for target cells by itself, and it fulfills all criteria considered as an IF. Analysis of its molecular constituents by SDS–PAGE showed that this substance is a mixture of Type I leukocyte IF (resistant to pH 2), with a mol wt of 25,000, and of a Type II immune IF (sensitive to pH 2), with a mol wt of 45,000. It was determined that both IF are not only antiviral but inhibitory of cell growth and that, whether crude or purified, they do indeed enhance the cytotoxicity of human NK cells.

In studies done on isolated lymphoid cell populations, the cells producing these IF in mixed cultures were Fc-receptor positive and surface Ig-negative lymphocytes, i.e., NK cells. The T-cells seem to be excluded as functioning in the production of IF in mixed cell cultures, and NK cells not only seem to interact with and to lyse within 4 hours IF-inducing target cells but also to be the lymphocytes responsible for the production.

In NK lymphocyte–target cell mixtures in which the target cell is a poor IF inducer, the addition of exogenous IF increases the cytotoxicity of the NK cells. When NK cells alone are pretreated, their cytotoxicity is enhanced and is greater than when the IF is added to the mixed culture. Such an enhancement is significant after 6 hours of preincubation and reaches a peak at 24–48 hours; thus it is an IF concentration-dependent effect. Studies with IF-producing lymphocyte populations suggest that it does not generate a new population of effector cells but rather enhances the activity of the mature NK cells. As opposed to its enhancement of NK cell activity, IF does not increase the efficiency of lymphocytes in mediating an ADCC toxicity, perhaps because IF stimulates a particular, nonimmune, recognition mechanism rather than lysis per se.

An additional recent and provocative observation is that in mixed cultures, IF appears to have an effect on *normal* target cells that is antagonistic to its effect on lymphocytes. When normal fibroblasts alone are pretreated, they become almost completely resistant to NK cell cytotoxicity, and this occurs whether or not NK cells have been preincubated with IF. Development of this resistance in the fibroblasts requires their ability to synthesize RNA and protein, as is true for the antiviral state also induced by the IF in normal fibroblasts; the concentration needed is of the same magnitude for both effects. Sensitivity to both IF-induced effects correlates strictly in various target cells. Although normal fibroblasts are susceptible to both effects, virus-infected or tumor cells may become antiviral but they remain unprotected and susceptible to lysis.

In all the in vitro and animal studies described in this section, it was ascertained that the active factors were, in fact, IF; in addition to other valid controls, parallel experiments were run with preparations of widely different levels of purity, including pure IF in the more recent in vitro studies; also, high titer anti-IF Ig was used in the neutralization of IF activity in control preparations.

A number of patients under treatment for viral diseases or neoplasms with HuLeIF preparations (with daily doses of 2×10^4–5×10^5 IU/kg body wt; sp act, 5×10^5–10^6 U/mg protein) have been monitored for their immune functions. No marked depression of humoral or CMI was observed, and only in a few instances was a reversible, modest depression of lymphocyte proliferation in response to PHA noted. Patients did become immunized against certain antigens present in the IF preparations (blood group, chick, and parainfluenza Type I antigens). Interestingly, however, some who had been treated for more than 3 months had marked decreases in complement fixation titers to several viruses and to mycoplasma. Apparently an inhibitor of the complement fixation reaction was present in their plasma that was associated with 7S immunoglobulin and which remains today only partially characterized. Complement fixation reactions returned to normal levels within weeks of the cessation of IF treatment.

Because human lymphoid cells behave like mouse cells in vitro with regard to their susceptibility to IF action, one can expect that immunosuppression or immunoenhancement could be observed in the clinic with the use of higher doses of human IF.

Some immunomodulatory activities have been known starting in 1972 and have already led to many speculations as to the possible role of IF in immunoregulation. The more recent studies on natural cell-mediated cytotoxicity point more specifically to their useful participation in a particular sector of the natural defense system which involves non-immune-type recognition. Such recognition apparently triggers IF production and lymphocyte-mediated nonspecific cytotoxicity against virus-infected and tumor cells; this cytotoxicity is enhanced by the IF produced which simultaneously serves to protect normal cells and thus increases the system's selectivity and efficacy. These studies and the conjectures to which they lead need urgent verification as part of a BRM Program and, particularly, if the role ascribed to IF is confirmed, a thorough evaluation must be made of the true effectiveness of the NK cell defense system in viral and neoplastic diseases.

The study of the many immunomodulatory effects of IF is important for an understanding of the mechanisms of action of the immune and IF defense systems and the interactions between them. Inasmuch as both are involved in various ways in the recognition of the defense against virus-infected and neoplastic cells, such studies are likely to produce valuable information for improved treatment of neoplastic, viral, immune, and perhaps other diseases.

TOXICOLOGY OF INTERFERONS

In contrast to the hyporesponsiveness of cells to repeated stimulation with any IF inducer, no hyporesponsiveness to the repeated induction of cells by IF occurs. Thus the IF phenotype can be maintained in cells simply through repeated administration of IF, i.e., through maintenance of a constant concentration in the cell's environment. For cultured cells, the level of the antiviral state can be monitored quantitatively and can even be let partially to decay and be restored rapidly to previous levels by the addition of IF to the growth medium.

In vitro, its continuous presence in the medium even at high concentrations and the resulting long-lasting, IF-induced phenotype do not cause any obvious harm to cells. All effects disappear rapidly as soon as IF is removed, and a normal cell growth rate resumes. However, whether a long-sustained induced phenotype would be deleterious to cells in vivo or the functioning of the organism as a whole may be another matter. In fact, deleterious consequences might be expected because nature has evolved complex and efficient mechanisms to suppress within hours IF production and its induced effects. The only cells known to be constitutive producers and, therefore, continuously express the induced phenotype are abnormal cells, which may be malignant, or laboratory artifacts obtained through mutagenesis, or both.

An investigator who made an early attempt at measuring the effect of prolonged treatment with IF in mice used doses comparable to those given in effective antitumor treatment of adult animals, administered daily, starting at birth, for 1 year. No toxic effects were observed; the animals grew and developed normally. However, in later experiments, newborn mice given daily injections of 50,000 IU mouse IF (i.e., 5×10^7 IU/kg body wt, corresponding to a daily dose of 2×10^8 IU in a human newborn, which is the equivalent of 100 times the present clinical dosage) failed to gain weight and died during the second week of life with extensive liver degeneration and other pathology. If treatment was stopped for 1 week, mice survived at first, then died 1 month later with severe glomerulonephritis.

In an attempt to understand and explain these pathological phenomena, scientists undertook studies on young mice infected with lymphocytic choriomeningitis virus. These mice develop high titers of endogenous IF and die of glomerulonephritis. If they are given anti-IF globulin, not only can they survive but the glomerulonephritis fails to develop, which points to their endogenous IF and *not* to the virus as the direct cause of the kidney disease which leads to their death. Thus the study of the toxicology of IF in animals should include studies that attempt to clarify whether IF given repeatedly and at high doses could be involved in the etiology of various diseases, such as the establishment of virus carrier states, the etiology or maintenance of chronic as opposed to acute infections, degenerative diseases, and autoimmune diseases.

Though not established, it is suspected from the above experiments and limited clinical experience (2 newborns treated with IF for congenital CMV infections who developed transient liver enzyme abnormalities) differences may be age related in IF toxicity. Because one of the major advantages of IF therapy in addition to its lack of gross toxicity is that it has neither mutagenic nor carcinogenic properties, careful preclinical and clinical toxicology studies should be done.

However, preclinical toxicology studies with substances such as IF should not be limited to the observation of gross toxicity appearing with increasing doses. They should include extensive preclinical and clinical work, accompanied by the monitoring of tissue and organ functions for a long period, particularly because IF treatment for cancer may require high doses given over months and perhaps even years. Such treatment could then be expected to have effects on immune functions, cell multiplication rates and differentiation, tissue regeneration, cellular enzymatic functions, and on the secretion of cellular products such as hormones. All these should be carefully monitored in patients within the IF component of the BRM Program.

PHARMACOLOGY OF INTERFERONS

Most of the evidence at hand today on the pharmacology of IF was obtained with HuLeIF (clinically and experimentally) and with mouse and rabbit IF (in humans and animals). All IF have a short half-life in blood circulation, irrespective of the route of inoculation. Injection of HuLeIF in rabbits and gibbons results in a half-life of about 75 minutes after im inoculation and of 10 minutes after a single iv inoculation. However, after steady-state iv infusion, the half-life is 2–4 hours. Maximum blood levels after it is given im are achieved within 8–10 hours, and doses given every 12 hours can provide nearly steady serum levels. Daily doses, which achieve less than 100 U of activity in the circulation, are presently being used clinically and have shown some activity.

Binding of IF to tissues seems the most logical explanation for their rapid clearance, inasmuch as they are distributed rapidly to various organs and tissues with no marked accumulation. Administered exogenous IF has poor penetration into certain body components e.g., the respiratory tract, cerebrospinal fluid, eye fluid, and the brain, although these tissues and fluids may contain high titer endogenous IF during natural viral infections. Urinary or intestinal excretion is minimal. In the lactating mouse, IF can be found in the milk and can protect weanling animals. Clearance of IF from the circulation is rapid at first and then decreases. The later slower rate of clearance may be due to IF eluting from cells after binding during the rapid clearance phase. Binding and subsequent elution have been documented in cultured cells.

Even when injected iv or intracisternally HuLeIF is well tolerated, and the side effects have been minimal. They consisted of a dose-related, mild febrile response, usually more marked after the first few injections, whether given by the im, iv, or sc routes. Myalgia and chills after each injection have been reported with daily doses greater than 1.7×10^5 IU/kg body wt. A feeling of fatigue and malaise is also cause for complaint, particularly from older and debilitated patients.

All the above-listed side effects have been observed with

preparations in which IF protein has been 0.1–1% of the total protein. The use of more purified preparations (10^7 IU/mg protein, 1–10% IF protein) has resulted in a significant decrease in the severity of side reactions and allowed daily administration of 5.10^5 IU/kg body wt.

Probably relevant to its pharmacological properties is a decrease and stabilization at a lower level of leukocyte, platelet, and reticulocyte counts during therapy. These effects of IF have been mild and reversible within days of the cessation of treatment; however, they may represent genuine reactions related to the ability of IF to slow cell multiplication. Also, fever may be directly due to IF because of its ability to induce the production of PGE. Elevated liver enzymes and decreased rate of weight gain in infants were similarly rapidly reversible effects but perhaps also directly related to its action.

The pharmacological characteristics of human fibroblast IF is almost completely unknown, although phase II-type clinical trials with this material have been initiated in Europe. Although in vitro it has all the properties of an IF, this type has been ineffective when given systemically to patients, even under those conditions in which HuLeIF had previously shown an effect, such as in the prophylaxis of viral infections in patients with renal allografts and in a trial in chronic hepatitis.

It is now obvious that the pharmacological properties of the 2 human Type I, i.e., leukocyte and fibroblast IF, are different. The clearance rate of the latter type appears to be much more rapid than that of the former IF. The fibroblast IF may not be detectable in the circulation after im inoculation; also, its high sensitivity to a factor present in extracts of human and mouse muscle tissue which can inactivate it within minutes has been reported. The clinical ineffectiveness following im injection is therefore easily explained and appears to be completely unrelated to the true biological activity of this material as an IF. As such, it may actually have desirable properties by virtue of its effectiveness in slowing the rate of multiplication of human osteogenic sarcoma cells in vitro, for which this form seems to have more binding affinity than does the leukocyte IF.

Regarding these differences between HuLeIF and fibroblast IF, interesting observations have been reported on the properties of the mouse material. This is routinely produced by cultured, fibroblast-type L-cells, and thus it has been assumed to be a fibroblast IF. Indeed, its rapid clearance and susceptibility to rapid inactivation by muscle extracts seem to confirm this assumption. However, when mouse leukocyte IF production was attempted (with mouse spleen cells) and the product compared with the mouse L-cell IF, no differences could be detected between the 2 substances. Both have the same molecular components and share common physicochemical and biological characteristics, including antigenic identity. Thus the mouse species seems to lack the equivalent of the HuLeIF and perhaps has a more primitive, less differentiated, IF system than is found in the human.

An intriguing parallel observation is that the doses of mouse IF required for an antitumor effect appear larger than are needed clinically. Researchers have routinely treated mice with about 20,000 IU of L-cell IF/day to obtain an antitumor effect. At this or even much higher doses, no reports confirmed tumor regressions in the mouse. Only a delay in tumor appearance or a slowing down in tumor growth with resulting prolonged survival and little or no effect on advanced neoplasia have been reported consistently. Although no real equivalency between mouse and human IF units exists and, moreover, transplantable mouse tumors have been selected for high malignancy, a dose of 20,000 IU/day in the mouse would correspond to 70×10^6 IU/day in humans, i.e., more than ten times the dosage with which tumor regressions have been obtained in advanced human cancer. Mouse IF appears less effective as an antitumor agent, perhaps because, like the human fibroblast IF it resembles in its pharmacological properties, it is rapidly inactivated in tissues.

Conduct of extensive additional pharmacological studies with the well-known and still highly impure leukocyte IF presently available would be useless. Investigators should try to produce it in amounts sufficient for its complete purification for a better use of limited resources, and thus they could establish its true pharmacological properties through testing it as a pure molecular species.

Also, pharmacological studies in animals and man should be performed as soon as possible with interferoids, the deglycosylated molecules which can be produced by human leukocytes in the presence of inhibitors of glycosylation. This information will be of paramount importance to scientists' plans for the production and clinical use of human IF through chemical synthesis or prokaryotes carrying the human *IF* gene. These methods will result in the production of a naked polypeptide and, should these prove unstable in vivo or undesirable for any other reason, work could start immediately on appropriate molecular modifications.

Finally, pharmacological studies will ultimately have to be done on IF modified and mainly fragmented either chemically or enzymatically. In the laboratory, such early modifications have already resulted in products with modified antiviral:growth-inhibitory activity ratios, indicating that analogs could be obtained with selected biological activities. However, modified IF will always have an immediate interest only if they also have desirable pharmacological properties in vivo.

As is true in toxicology studies, the pharmacology of IF must include an important research component in that, in addition to providing information immediately relevant to activity and safety in clinical trial with the crude materials presently available, it has to provide important information on the basis of which the development of future production and purification methods can be planned that will result in either IF polypeptides, active fragments, or modified molecules.

STUDIES IN ANIMAL MODEL SYSTEMS

Much additional work in animals is needed now in the following major areas: 1) pharmacology of IF and modified IF; 2) mechanisms of action of IF as antitumor agents, i.e., the relative importance of direct and host-mediated antitumor effects of IF, and the effects of IF on carcinogenesis and metastases; 3) development of the best mo-

dalities of antitumor treatment, particularly in combination therapies, and the development of chemoprevention with IF; and 4) studies in nude mice.

Pharmacology and Toxicology

Whatever toxicity IF treatment may have at any dose and in any species will not be the direct result of the administration of a few micrograms of a physiological protein but the result of its biological activity. Human IF are inactive biologically in most heterologous species (as they are in the mouse) and are always antigenic in them. Therefore, their possible toxicity to the human cannot be evaluated in animals nor even in other primates. Animals will have limited usefulness in toxicology and pharmacology studies involving human IF; only their homologous IF can be validly and completely tested in them in these respects.

On the other hand, animals have been useful in the past for certain studies of the rate of clearance of HuLeIF. Similar studies should now be done with the human fibroblast IF and with the human Type II, and, when they become available, human interferoid and modified IF (these will subsequently also need clinical pharmacological evaluation).

Animals, and particularly the mouse, will be uniquely beneficial as models for the evaluation of the in vivo biological activity and the pharmacological properties of their homologous interferoids and modified IF. Initially, the deglycosylated mouse interferoid already available should be studied immediately for its stability, clearance rate, and activity in vivo.

Mechanism of Action of Interferons as Antitumor Agents

A number of animal species could be useful in these investigations. However, work with any species other than the mouse would be considerably more onerous: On a unit basis, the production of most animal IF is as, or more, expensive than that of human and mouse material, for both of which effective preparative scale procedures for production and partial purification are available; complete purification has been achieved for both. Moreover, the properties of the IF of other species are not well-known, and even the mouse type has only recently been studied pharmacologically in some detail. All animal IF may be different from those of the human. In addition, with rare exceptions, such as osteogenic sarcoma in the Beagle dog and some tumor models in the rat, species other than the mouse do not provide tumor models significantly better than those of the mouse. Other animals are mostly larger and therefore require more IF or treatment, and experimentation with them takes a longer time. For all these reasons, the bulk of the experimental animal work required for the development of clinical IF therapy will, by necessity, have to be done in the mouse.

The advantages the mouse presents as an experimental animal are obvious, including the existence in that species of an athymic mutant, the nude mouse, which represents the only genetically determined immunodeficiency known except in man. Also, despite their limitations, the mouse tumor models have been useful in the past in predicting the efficacy of chemotherapeutic drugs in man. A number of questions still unanswered that could be addressed immediately in the mouse are: 1) the importance of direct and host-mediated antitumor effects of the IF, and 2) the effects of IF on carcinogenesis and metastases.

Importance of Direct and Host-mediated Antitumor Effects of Interferons

Tumor cells would be required that are capable of growing both in vitro and in vivo, so that they can be treated with IF and observed either in culture, alone, or in combination and various host cells (lymphocytes and macrophages, untreated or pretreated with IF) and in vivo. Several mouse tumor cell lines fulfill these criteria. These are, among others:

Meth A cells.—Derived from BALB/c mice treated with MCA, these cells can grow as suspension cultures in the ascites form and subcutaneously in syngeneic nude mice. They can be recovered from the peritoneal cavity of animals as a 99% viable cell suspension. Also, 10–100 Meth A cells can cause a tumor in intraperitoneal and subcutaneous locations. They lend themselves exceedingly well to quantitative studies.

L1210 cells.—These malignant lymphoid cells have already been studied with IF to which they are sensitive. L1210*R* cells resistant to its antiviral and growth inhibitory action have been selected. The availability of L1210*S* and L1210*R* cells (either susceptible or resistant to direct IF action) offers unique opportunities for experimentation in which the host-mediated effect can be clearly separated from direct IF effects.

Osteosarcoma cells.—Transplantable mouse osteosarcoma cells have been derived from a radionuclide-induced osteosarcoma of the mouse. These cells grow in vitro and in vivo and metastasize; they are susceptible to inhibition by IF in both situations.

Lung adenocarcinoma cells.—Strain A mice are highly susceptible to the induction of lung tumors when given iv injections of MCA. Tumor cells can be explanted and grown in culture.

None of the above cells are believed to require the full expression of a viral genome for their multiplication, although C-type particles are associated with them, as with all mouse tumor cells.

The above studies should include research at the cellular and subcellular level, on both the direct and host-mediated effects of IF, including, at this time, the confirmation of the role of NK cells and of the IF they produce as a result of their interacting with virus-infected or tumor cells.

Effects of Interferons on Carcinogenesis

Few studies have been reported on the effect of IF on the process of carcinogenesis, although in 2 in vivo investigations, IF has been effective in delaying or inhibiting, or both, tumor appearance entirely, and it can also enhance the production of aryl hydrocarbon hydroxylase induced in cultures by benzanthracene.

Strain A mice, sensitive to induction of tumors by carcinogenic stimuli, respond with the development of lung

tumors later in life in all the animals. A single injection of 0.5 mg MCA results in an average number of nearly 50 tumors/animal. The time required for tumor development (100 days in adult mice) can be shortened by inoculation of newborn mice. Numbers of tumors can be quantitated precisely by fixation and staining of the whole lung, in which tumors can be counted as white nodules. Meth A mouse tumors have already been shown to be sensitive to inhibition by mouse IF.

Inhibition of carcinogenesis can also be studied in osteogenic sarcoma tumors induced by radionuclides that appear predictably in numbers that are closely related to the radiation dose administered. In a single recent report, mouse IF Types I and II have been active in this system as well.

Undoubtedly, other systems exist, e.g., chemically inducible bladder, colon, and liver tumors in mice or rats, or both, all of which would allow one to investigate what specific event in the carcinogenic process IF can inhibit: initiation or promotion, or both. Treatment with IF inducers or the crude exogenous material has radioprotective properties. The investigators whose research led to this intriguing observation were stimulated by the finding that all groups of substances with demonstrated radioprotective ability (aminothiols, vasoactive biological amines, and bacterial endotoxins) were IF inducers. The mechanisms of inhibition of radiation-induced carcinogenesis should be reexamined.

As far as virally induced oncogenesis is concerned, much more work could expand the early observations that mouse IF inhibits oncogenic transformation and cellular DNA synthesis stimulated by DNA-containing oncogenic viruses (polyoma and SV40) thus aborting tumor induction in vivo. Once a viral genome is integrated, virus-specified gene products are resistant to IF action. Thus, once expressed, the T-antigen of SV40-transformed cells remains unaffected by IF treatment. These experiments were done in vitro. However, whether such cells are also resistant to an antitumor effect in vivo has not been tested. In view of the susceptibility of L1210*R* cells, this and other DNA virus-induced tumor systems should be reexamined.

The mechanisms which result in an inhibition of tumor growth induced by oncoviruses remain to be clarified, including the suppression by IF of the lymphomas associated with oncovirus induction during the GVH reaction in mice.

When IF are available in quantity, treatment could also conceivably be exploited for chemoprevention of carcinogenesis, whether in individuals at high risk from chemical or physical hazards or those with a genetic propensity to the development of cancer (familial polyposis, retinoblastoma, xeroderma pigmentosum, neurofibromatosis, etc.).

Given the present interest in the process of carcinogenesis and the importance of the resources allocated to its study, a tool such as IF, which can suppress carcinogenesis, should be used in these investigations. As a research tool, it can help gain valuable insights and contribute to conceptual advances. It could also develop into a practical method of tumor prophylaxis for individuals at high risk.

Effects of Interferons on Metastases

Metastatic potential is an all important parameter of neoplasia. In 2 reports, mouse IF has significantly decreased the metastatic spread of the Lewis lung carcinoma and that of a transplantable osteogenic sarcoma of the mouse. This evidence is important in three respects because it suggests that 1) IF given ip can be effective at distant sites, e.g., the lung; 2) it can reduce the development of metastases by 2 tumors, different histologically, and probably also in their original etiology; 3) it provides evidence which lends support to observations made in patients that have been difficult to interpret. In the Swedish trial in human osteogenic sarcoma, patients who were given IF had fewer metastatic recurrences in the lung than those not so treated.

Obviously, confirmation and expansion of studies in the mouse to other tumor types are important as are thorough investigation and clarification of the mechanism(s) of the antimetastatic effect of IF.

Several mouse tumors lend themselves to such investigations. The Lewis lung carcinoma of C57BL mice, which was already mentioned, grows rapidly subcutaneously and develops macroscopic pulmonary metastases that can be counted with ease within 3 weeks after tumor cell inoculation. Further studies should be done with it, and the conditions should be defined under which complete suppression of metastases can be obtained. The same should be attempted in the radionuclide-induced, transplantable mouse osteogenic sarcoma.

The B16 melanoma would also lend itself ideally to similar quantitative research; its cells grow rapidly in vivo and in vitro. Measured numbers of cells can be inoculated locally into the ears of mice, and primary tumors can be removed easily after specific intervals. The primary tumors give rise to metastases which can be counted in the lung even without prior fixation and staining. Moreover, clones of B16 tumor cells have been selected which have both high and low metastatic capacities to either the lung or the brain. They "home" on the organ for which they have been selected. This characteristic could provide a basis for valuable research on the activity of IF in these particular organs, i.e., its relative ability to reach them. Here, too, conditions that suppress metastasis to the maximum extent should be sought and defined.

In these 3 systems, as well as others that might be found suitable, it is important that studies of metastatic spread in animals be accompanied by those at the cellular and subcellular level on the effects of IF in vivo and in vitro on cells of the primary tumor, metastases, and the host, particularly the host's B- and T-cells, NK cells, and macrophages.

For example, certain tumor-cell products, such as angiogenesis factor and plasminogen activator, have been broadly associated with cancers. Some have speculated that they might contribute to the tumor cell's metastatic potential through their effects on the normal tissues surrounding growing tumors. Results of a recent study have indicated that mouse IF inhibits secretion of the plasminogen activator by tumor cells. This effect must first be confirmed in other tumor models, so that we can

establish whether it is a general occurrence; should this be so, further attempts should be directed toward establishment of whether 1) plasminogen activator inhibition in vitro changes the behavior of IF-treated cells when in vivo, and 2) an inhibition can be obtained in vivo that can also be measured in vitro. If so, does this modify other tumor cells behavioral characteristics, such as potential for colony formation in semisolid media, cell saturation density, etc.? The B16 melanoma cells, which are readily grown, would appear most appropriate for studies of this kind.

For the in vivo study of metastasis, the Beagle dog radionuclide-induced osteogenic sarcoma, which seems to be an excellent model of the human disease, could be used in parallel to the osteogenic sarcoma of mice.[4]

The above work, which can be done under conditions that allow quantitative studies and the monitoring of IF effects on metastasizing and host defense cells, should be pursued intensively in as many metastatic tumor models as lend themselves to such investigation.

Development of Best Modalities of Antitumor Treatment

In mouse tumor systems in which the best modalities of treatment could be evaluated quantitatively through the use of cell counts, susceptibility of tumor cells to IF treatment correlated (inversely) with tumor load; also, the same tumor cells were more susceptible if inoculated ip as cell suspensions, rather than sc, and thus were made to grow as solid tumors. Because the IF was always administered ip, one can conclude that an intimate contact between the IF and the tumor cells appeared desirable. However, we now know that mouse IF is rapidly and actively inactivated in mouse tissue extracts and that solid mouse tumors located at distant sites might be less susceptible because less material reached them. The more stable HuLeIF may be expected to be more effective generally, especially against solid tumors at distant sites.

In the mouse, syngeneic tumors have been susceptible to inhibition by mouse IF irrespective of their tissue of origin, i.e., whether they were transplantable or had originated in the mouse and, if in the mouse, whatever the external stimulus was that originally brought about the malignant transformation or whether the tumor was spontaneous. Mouse IF certainly has a broad spectrum antitumor activity.

In man, the limited evidence currently available indicates that tumor load and general status of the patient appear to be more important determinants of the susceptibility of a tumor than its histological type, cell kinetics, or degree of malignant potential, or whether the neoplastic disease is a leukemia or a solid tumor. The HuLeIF may be more effective against tumors of the lymphoid system (multiple myeloma and lymphoid cell leukemias appear to respond particularly well) but it appears active also in several other benign and malignant diseases. Therefore, the same situation appears to prevail in mice and humans, i.e., apparently, IF treatment is effective against a broad range of tumor types.

[4] Dog IF can be readily produced by conventional methods from leukocytes and fibroblasts.

Because of these emerging similarities between the mouse and the human species, and within limitations due mainly to the more rapid in vivo inactivation of the mouse IF, animal models and the mouse in particular could provide valuable guidelines to clinicians concerning the relative dosage of IF needed in the treatment of their cancer patients.

Using IF alone, physicians could explore various regimens of administration systematically, such as pulse treatment versus continuous treatment. For example, intermittent treatment to the mouse allows high doses to be administered without elicitation of undesirable immunosuppression, whereas similar daily doses are markedly immunosuppressive and thereby negate treatment benefit.

More importantly, in the development of the most effective combined therapies, the systematic exploration of the various combinations and permutations possible can best be done in animals and much more rapidly. If synergistic effects are observed, animals allow the rapid exploration of IF dose reduction and the extent to which this can be done without losing the contribution IF makes to the combined therapeutic effect.

Synergistic (because more than additive) effects have already been observed in several combinations tried in the mouse. The following cytoreductive methods were successfully added to IF therapy: surgery in mammary carcinoma; BCNU in transplanted LSTRA leukemia; CPP or Cytoxan or vincristine in the spontaneous AKR leukemia; adriamycin in mammary carcinoma; and a mild immunostimulator, Isoprinosine, in sarcoma 180.

Thus almost any transplantable syngeneic tumor cell and any spontaneous tumor occurring with a high incidence in any strain of mice can be used in experiments of this kind; both types of tumors should be studied. However, for optimal relevance to the human situation, perhaps tumors requiring the production of mature virions for their continuous growth (e.g., Friend leukemia) should not be regarded as adequate models.

Cytoreductive therapies to be added to treatment with IF should include: surgical ablation of the primary tumor in metastasizing systems, chemotherapy with drugs known to be effective *and* ineffective on a particular mouse tumor, radiotherapy, immunotherapy, and IF inducers.

Studies in Nude Mice

Mice homozygous for the mutant *nu* gene or nude mice have no functional T-cells and a B-cell immune system which, though present, is abnormal. They seemed to offer a unique opportunity for the propagation of human tumor cell lines and even for the growth of human tumor biopsy material in a xenogenic situation; thus they allow the direct screening of potential therapeutic agents, including IF, on human tumor nodules obtained directly from the patient before treatment.

Numerous investigators attempted to explant various surgical tumor fragments into nude mice, and sometimes serial passages were made. Various human tumor types, each sometimes represented by multiple isolates, have been maintained in these mice. Well-authenticated human tumor

cell lines propagated in vitro are also available which have been adapted to grow in nude mice.

However, far from all such attempts have been successful, and some human tumors were found never to "take." It is now recognized that nude mice do, in fact, actively reject certain xenogeneic cells and that the defense system responsible may be, as in fully immunocompetent animals, the NK system (*see* Immunomodulation by Interferons).

Tumor cell lines chronically infected by viruses are more effectively rejected by nude mice than their noninfected counterparts. This enhanced rejection can be stimulated by various chronic viral infections in target cells, and the response is nonspecific because animals stimulated by 1 virus more effectively reject target cells infected by many others. It was determined that the mediator of this nonspecific response is IF, produced, as in the tumor rejection mechanism operative in normal animals, by small bone marrow-derived Fc receptor positive, non-T, non-B, effector lymphocytes, i.e., the NK cells. Finally, the administration of exogenous mouse IF to tumor-bearing nude mice markedly enhanced the rejection mechanism against human or other xenogeneic tumors, whereas the administration of anti-mouse IF globulin markedly depressed the ability of nude mice to reject such tumors, virus infected or not. If the anti-mouse IF globulin was given shortly before a tumor cell transplant, the take of xenogeneic tumor cells was greatly facilitated.

We shall have to wait to see if repeated treatment of nude mice with this globulin can be put to practical use in helping to maintain and passage human tumors that would otherwise be rejected.

However, in unrelated experiments it was observed that human exogenous IF administered to nude mice bearing human tumors also inhibited the growth of the human tumor cells.

These apparently paradoxical results can perhaps be reconciled in the light of the ability of IF to operate *directly* on homologous tumor cells only and *indirectly* through host-mediated rejection mechanisms on heterologous tumor cells. It is entirely possible that a direct growth-inhibitory effect (even if modest) exerted by human IF on the human tumor cells could have tipped the balance in favor of rejection by the mouse NK cell system which produces its IF anyway, in response to the presence of human tumor cells. These interesting observations should rekindle interest in the nude mouse as a xenogeneic host for human tumor cells. With no serologic cross-reactivity between the human and mouse IF, and only negligible ($<1\%$) cross-species activity of the human IF in the mouse, we might effectively use anti-mouse IF globulin-treated nude mice to grow human tumor explants, which could still be tested for their susceptibility to the direct growth-inhibitory effect of various concentrations of human IF alone or in combination with cytoreductive therapies.

Should the above findings be verified, they would open new opportunities for scientists to exploit the nude mouse as a xenogeneic host for human tumor cells, whether these be continuous cell lines or primary explants, for the screening of potential antitumor agents including IF. These opportunities should be actively pursued because they may allow us to observe effects on human tumor cells in the absence of significant host-mediated rejection mechanisms, i.e., to study their direct effects in vivo. Such an opportunity is particularly important for the IF, the antitumor effect of which is complex and not well understood.

The study of the IF system in vivo in various mouse strains, including the nude mouse and in other species whenever these offer suitable models, with highly purified or pure IF preparations and high titer anti-IF globulins, will produce a wealth of new information of both fundamental and practical importance.

Conceptually, such research will clarify the natural role of the IF defense system and whether it can participate in certain pathogenic processes; also clarified will be the mechanisms of its antitumor action including the importance of its direct and host-mediated aspects, and the nature of the nonimmune recognition mechanisms operative in these mediated aspects. Therefore, such studies are likely to have an impact on present-day thinking concerning the etiology, pathogenesis, and therapy of cancer and other diseases.

At the pragmatic level, animal experimentation should provide useful guidelines for a rational approach to treatment with IF alone or in combination therapies and not only for cancer therapy. Such research might save time and expense in the process of developing new modalities of treatment for human cancer.

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SECTION IV

Thymic Factors and Hormones

INTRODUCTION

A number of investigators have now succeeded in isolating and purifying factors with thymic hormone-like activity from thymus and blood; these preparations are in various stages of characterization. Among the preparations, thymosin fraction 5, THF, TP, TFX, and FTS are the most thoroughly studied and best characterized. Thymosin fraction 5 contains a number of active peptides with mol wt ranging from 1,000 to 15,000, several of which have now been purified to homogeneity and sequenced. Four hormones have been synthesized (thymosin α_1, thymosin β_4, TP II, and FTS, with mol wt of 3,108, 4,902, 5,562, and 857, respectively). These thymic peptides appear to be chemically distinct and act selectively on the development of immune functions.

The importance of these thymic hormones as biological modifiers lies in the potential usefulness of chemically defined hormones that will specifically increase host resistance by accelerating the maturation of T-cells from precursor cells, especially in patients with cancer. This accelerated maturation would be advantageous in helping to reverse the significant immunosuppressive effects of chemotherapy, radiotherapy, and surgery, and of the tumor itself. Preliminary trials with thymic hormones look promising. These agents, as described in the following report, have important beneficial effects in children and adults with immunological disorders, whether congenital or associated with malignant or collagen vascular diseases.

Thymosin fraction 5, the thymic hormone preparation used in the larger number of clinical trials to date, is proving to be of significant benefit in the treatment of children with primary immunodeficiency diseases, such as DiGeorge syndrome, chronic mucocutaneous candidiasis, Wiskott-Aldrich syndrome, and other immunodeficiencies associated with thymic hypoplasias and dysplasias. In clinical trials now entering the seventh year, significant improvements have been observed in T-cell numbers and function, decreased infections, increased weight gain, and overall clinical improvement (*1–12*). Recently, Wara et al. (*7*). Barrett and co-workers (*8*), and Sharp and Peterson (*9*) have reported that, in the DiGeorge syndrome, thymosin treatment appears safe and efficacious and is indicated in patients whose lymphocytes respond to thymosin fraction 5 in vitro. Studies with THF have shown that T-cell function is restored in children with subacute sclerosing panencephalitis (*13*) and rheumatoid arthritis (*14*).

In cancer patients, phase I toxicity studies and a limited number of phase II trials in patients with far advanced neoplasms have been conducted over the past 6 years with thymosin (*13–19*), TFX, (*20, 21*), THF (*22, 23*), and TS (*24*). The thymic factors appear to be nontoxic and to induce increased T-cell numbers and function.

The first randomized phase II trial of thymosin, designed by Chretien, was conducted by Cohen and his colleagues at the Veterans Administration Medical Center, Washington, D.C. The trial was a 3-arm study of 55 patients with oat cell carcinoma of the lung. Thymosin fraction 5 was given for 6 weeks in conjunction with intensive chemotherapy. The results of this trial (*15–17*) indicated that 60 mg thymosin/m^2 given for 6 weeks significantly prolongs survival in patients who had eradication of all detectable tumors by chemotherapy (median survival with chemotherapy plus placebo was 240 vs. 450 days after chemotherapy plus thymosin fraction 5). Further analysis of this study showed that thymosin had no detectable direct antitumor effect, but it may have ameliorated the immune defects due to the cancer or the chemotherapy because the patients who benefited most from thymosin were those with relatively low immune reactivity.

This lung cancer trial with thymosin is the first positive controlled trial involving a thymic hormone preparation, or, for that matter, any BRM, in oat cell carcinoma. If this study can be confirmed, it will be a major step forward in the treatment of this disease.

The ultimate significance of the thymosins and other thymic factors in cancer treatment should be in the provision of a means of safe augmentation of specific lymphocyte functions in patients with diminished immunity. In anergic cancer patients, the thymic hormones may be important as adjuncts to conventional modalities of therapy by increasing T-cell function in response not only to tumor cells, but to pathogens; thus they reduce the high incidence of infection that often accompanies treatment of cancer with conventional modalities.

The positive clinical trials to date with the partially purified thymosin fraction 5 in children with immunodeficiency diseases, the positive oat cell trial of Chretien and his colleagues, and the reported positive studies with THF and other thymosin fraction-like preparations offer a strong rationale for the rapid confirmation of the clinical studies and expansion of the basic research programs with the goal of chemical and biological purification and characterization of the active peptides that comprise thymosin fraction 5 and other thymic factors. Eventual utilization of the synthetic thymic peptides should provide clinicians with novel approaches to the treatment or prevention of various diseases characterized by or accompanying thymic malfunction, including cancer.

Our purpose in this report is to present an update of the current state of our knowledge of the chemistry, biology, and clinical applications of the well-characterized thymic preparations. In addition, areas for priority development will be proposed with regard to new and large-scale clinical studies and further biochemical and pharmacological characterization of the thymic hormones.

CLINICAL TRIALS

Studies with Thymic Hormonal Preparations

Most clinical studies of thymic preparations have been reported with 3 products, i.e., calf thymosin fraction 5 (*1–19*); THF (*22, 23, 25–28*); and thymosin fraction 5-like preparations, such as TFX (*20, 21, 29*), TS (*24, 30*), and porcine (*31*) and bovine (*32, 33*) fractions. Most recently, a phase I trial with synthetic thymosin α_1 was performed by Ommaya (National Institute of Neurological and Communicative Disorders and Stroke) and Chretien (NCI) in patients with brain tumors (Ommaya AK: Personal communication).

These clinical studies included in vitro examination of the ability of the thymic preparation to enhance formation by separated PBL of rosettes with sheep erythrocytes (E-rosettes) or response to antigens, mitogens, and allogeneic cells after either incubation of the cells in vitro with the fraction to be tested or administration to human subjects by injection of the preparation. In addition, the responses of treated individuals to recall antigens, with delayed skin hypersensitivity as a model, have been described.

The clinical studies with thymic preparations reported [with the exception of the oat cell trials of Lipson et al. (*16*), Chretien and associates (*17*), and Schafer and coworkers (*18*)] have not included critical, randomized "protocols." The small numbers of patients available and the ethical considerations have precluded randomized clinical trials in the pediatric studies conducted to date. However, investigators designed the earlier trials and certain of the continuing reports to obtain a broad, early perspective of the possible efficacy of thymic products in human diseases, notably those with impaired immunological components.

Astaldi and his colleagues reported (*34, 35*) that when patients who had combined immunodeficiency or single cases of either the DiGeorge syndrome or a cellular immunodeficiency due to lymph nodal mycosis received injections of thymosin, the treatment induced the appearance of SF in their blood. The thymosin treatment also resulted in the disappearance from the blood of lymphocytes that respond in vitro to the SF. The authors hypothesized that thymosin acts on as yet unknown organs or cells to induce production or release of this factor. No objective evidence was obtained for clinical benefit to the patients resulting from thymosin administration.

Most of the reported trials with thymosin, THF, and thymosin-like preparations in patients with various clinical entities cannot be evaluated critically at present because either the number of treated patients with a given clinical syndrome was too small for analysis of the finding or the subjects receiving placebos were not included. Also, in most instances, the nature of the preparation or preparations administered was known to the investigators. Nonetheless, from a wide variety of clinical observations and the well-controlled thymosin study of Chretien and his colleagues, the available information suggests the following:

1) Thymic hormone replacement therapy may be efficacious in children with syndromes attributable to failure of maturation of a normal thymus gland, i.e., thymic aplasia or dysplasia, but not in the untreated newborn with combined immunodeficiency due to congenital absence of the thymus.

TABLE 4-1.—*Summary of pediatric patients entered in thymosin factor 5 trials, 1974–79*[a]

Type of disease	No. of patients	No. continuing	No. of patients discontinued				No. completed	No. transferred	No. died
			Failure to respond	Side effects	Non-related	Thymus transplant			
Thymic hypoplasia with adenosine deaminase deficiency	2	2							
Thymic hypoplasia without adenosine deaminase deficiency	6		2						4
Thymic hypoplasia with varicella	1						1		
Severe combined immunodeficiency	5	2	1	2					
Combined immunodeficiency	16	2	13						1
Secondarily induced immunodeficiency	1								1
Cellular immunodeficiency with mycosis	1		1						
Cellular immunodeficiency	1					1			
Ataxia-telangectasia	9	4	1	1	2				1
Chronic mucocutaneous candidiasis	7	1	4		1				1
Congenital agammaglobulinemia	1		1						
Wiskott-Aldrich syndrome	11	3		5	2			1	
Partial T-cell defect	3	1							2
Atopic eczema	2	1			1				
Cartilage hair hypoplasia with varicella	1						1		
Cartilage hair hypoplasia	3		3						
Subacute sclerosing panencephalitis	1		1						
DiGeorge syndrome	9	1	1		1	1	2		3
Histiocytosis X	1		1						
Secondarily acquired hypogammaglobulinemia	1			1					
Total	82	17	29	9	7	2	4	1	13

[a] Information was supplied by Clifford Joseph, M.D., Associate Director of Medical Research, Hoffman-La Roche, Inc., Nutley, New Jersey.

2) Therapy with preparations exhibiting thymic hormone activity may be useful as adjunct or supportive treatment of clinical neoplasms by their reconstituting the host immunological deficiencies and by increasing immunological and hematological tolerance to contemporary oncological therapy by counteracting the immunopoietic and myelopoietic suppressive side effects of this regimen.

3) The limited reports of efficacy of thymic preparations in infections on some clinical syndromes require extended and more critical clinical trials.

4) The efficacy of these products in treatment of diseases characterized by an autoimmune component has not been evaluated.

Summary of Phase I and II Trials With Thymosin

Primary Immunodeficiency Diseases

Over 80 children received thymosin fraction 5 for a variety of primary immunodeficiency diseases (*1–10*). Up to 400 mg thymosin/m^2 was given to these patients by injection usually daily for 2 to 4 weeks, then once/week for periods of over 6 years; most of the patients received 60 mg thymosin/m^2 sc. As indicated in table 4-1, 17 patients continued on treatment. To date, 9 of the first 82 patients were discontinued from treatment because of the side effects, as summarized in table 4-2. No evidence of liver, kidney, or bone marrow toxicity due to thymosin administration was observed in this group.

TABLE 4-2.—*Side effects of thymosin observed in pediatric patients receiving thymosin fraction 5*[a]

Type of disease	Type of side effect	No. of patients
Severe combined immunodeficiency	Encephalopathy (acute)	1
	Progressive thrombocytopenia and eosinophilia	1
Ataxia-telangectasia	Suspected lymphoma or lymphoreticular cancer[b]	1
Wiskott-Aldrich syndrome	Wheezing	1
	Severe local reactions	1
	Severe local reaction; increased fibrin split products[b] in serum and bleeding diathesis	1
	Wheal and severe local reaction	1
	Swelling and pain of entire arm	1
Secondarily acquired hypogammaglobulinemia	Nosebleeds[b]	1

[a] *See* footnote *a*, table 1.

[b] Serious doubt exists that these represent true reactions to thymosin.

A summary of the biological effects of thymosin in the first 17 pediatric patients (table 4-3) revealed a close correlation between the number of patients who responded in vitro in the E-rosette assay and those who also responded in vivo with increased absolute T-cell numbers and clinical improvement.

In a more recent study of 17 immunodeficient patients in whom other forms of therapy either were unsuccessful or could not be utilized, Wara and Ammann (*5*) reported significant clinical improvement in patients with various thymic-dependent immunodeficiency diseases who were treated with thymosin. All had evidence of enhanced lymphocyte formation of T-cell rosettes or enhanced lymphocyte function, or both, as assayed in MLC following incubation with thymosin fraction 5. Results of E-rosettes and MLC responses obtained before therapy and 6 months later in this group are reported in tables 4-4–8. A description of their clinical responses are as follows:

Four patients with chronic mucocutaneous candidiasis received thymosin (table 4-3). The first, a 13-year-old female, had diffuse candidiasis of her skin and mucous membranes, no associated endocrinopathy, decreased T-cell numbers, a nonreactive *Candida* skin test, and depressed lymphocyte response in MLC and to *Candida* antigen. The patient had enhanced T-cell rosette formation and lymphocyte response in MLC with thymosin incubation. After 6 months of therapy, her cutaneous candidiasis was markedly cleared and she had a 5-kg weight gain, normal total number of T-cells, and a normal lymphocyte response to MLC. A skin test for *Candida* remained negative as did lymphocyte response to it. Three additional patients (#2–4) with chronic mucocutaneous candidiasis were treated; none of these had a significant increase in T-cell rosette formation with thymosin incubation, and none had a substantial increase in T-cells following thymosin therapy. However, all (#2–4) had an increase in lymphocyte response in MLC with thymosin incubation, and the 1 patient evaluated 6 months after initiation of therapy had an increase in MLC response. However, the other 2 patients did not receive 6 months of therapy.

Three patients with ataxia-telangiectasia received thymosin for 6 months to 1 year. Prior to therapy, all 3 had increased infections and 1 (#6) had North American Burkitt's lymphoma. Two of the 3 had decreased total numbers of T-cells; none had enhanced T-cell rosette formation with thymosin incubation. All 3 had depressed lymphocyte response to PHA and in MLC. Following 6 months of thymosin therapy, 2 of 3 had depressed T-cell numbers and 1 had a transient increase (table 4-4). Only 1 patient (#7) had a significant increase in lymphocyte response to PHA and in MLC. The patient with North American Burkitt's lymphoma died with disseminated lymphoma 6 months after the initiation of therapy; the other 2 remained clinically stable during therapy.

The 3 patients with Wiskott-Aldrich syndrome who received thymosin fraction 5 for 6 to 30 months (table 4-5) had significant systemic infections, infected eczema before therapy was initiated, decreased total numbers of T-cells, enhancement of T-cell rosette formation with thymosin incubation, and depressed lymphocyte responses in MLC. One patient had a depressed lymphocyte response to PHA and 2 were normal. After 6 months of therapy, all had

TABLE 4-3.—*Thymosin therapy in patients with chronic mucocutaneous candidiasis*[a]

Patient No.	Age, yr	Sex	Total No. of T-cells[b]		PHA[c]		MLC[d]	
			Before	After	Before	After	Before	After
1	13	Female	574(935)[e]	1,340	40,327/822	35,710/170	4,029/746	6,474(8,860)/154
2	49	Male	1,890(1,972)	2,415	32,513/168	36,513/168	14,827(32,548)/309	27,749/149
3	11	Male	1,379(1,602)	1,510	44,437/129	ND[f]	4,226(7,040)/146	ND
4	18	Female	780(810)	827	24,755/375	ND	4,720(8,678)/241	ND

[a] *See* (5).
[b] Values represent total lymphocyte count × percent T-cell rosettes; normal value = 900/mm³.
[c] Values represent in vitro lymphocyte response to PHA: stimulated cpm/resting cpm. Normal value = 42,000 cpm.
[d] Values indicate in vitro lymphocyte response to allogeneic cells: stimulated cpm/resting cpm. Normal value = 7,000 cpm.
[e] *Numbers in parentheses* = thymosin incubation in vitro.
[f] ND = not done.

TABLE 4-4.—*Thymosin therapy in patients with ataxia-telangiectasia*[a]

Patient No.	Age, yr	Sex	Total No. of T-cells[b]		PHA[c]		MLC[d]	
			Before	After	Before	After	Before	After
5	6	Male	737(927)[e]	724	4,669/240	2,547/195	3,979/319	1,518/85
6[f]	5	"	1,351(1,396)	2,214	1,485/348	605/505	4,674/988	1,586/702
7	6	"	739(739)	528	814/230	14,081/337	555(2,065)/149	1,735/658

[a] *See* (5).
[b] *See* footnote *b*, table 3.
[c] *See* footnote *c*, table 3.
[d] *See* footnote *d*, table 3.
[e] *See* footnote *e*, table 3.
[f] Patient had North American Burkitt's lymphoma.

TABLE 4-5.—*Thymosin therapy in patients with Wiskott-Aldrich syndrome*[a]

Patient No.	Age, yr	Sex	Total No. of T-cells[b]		PHA[c]		MLC[d]	
			Before	After	Before	After	Before	After
8	2	Male	414(622)[e]	654	57,226/460	25,218/397	2,368(3,430)/420	479/162
9	3	"	980(1,642)	1,260	63,205/730	81,346/486	2,248/973	5,213/165
10	12	"	252(408)	825	3,140/147	63,150/452	726/54	14,568/1,083

[a] *See* (5).
[b] *See* footnote *b*, table 3.
[c] *See* footnote *c*, table 3.
[d] *See* footnote *d*, table 3.
[e] *See* footnote *e*, table 3.

TABLE 4-6.—*Thymosin therapy in patients with primary immunodeficiency*[a]

Patient No.	Age	Sex	Diagnosis	Total No. of T-cells[b]		PHA[c]		MLC[d]	
				Before	After	Before	After	Before	After
1	5 yr	Male	Severe combined immuno-deficiency	1,546(1,542)[e]	1,619	2,763/146	7,297/2,063	869(850)/516	3,477/2,466
2	1 mo	Female	DiGeorge syndrome	343(350)	214	68,271/413	7,184/625	1,708/213	1,233/110
3	1 mo	Male	DiGeorge syndrome	398(1,417)	2,751	20,533/420	59,401/233	771(4,964)/111	25,871/5,016

[a] *See* (5).
[b] *See* footnote *b*, table 3.
[c] *See* footnote *c*, table 3.
[d] *See* footnote *d*, table 3.
[e] *See* footnote *e*, table 3.

TABLE 4-7.—*Thymosin therapy in patients with primary immunodeficiency*[a]

Patient No.	Age, yr	Sex	Diagnosis	Total No. of T-cells[b]		PHA[c]		MLC[d]	
				Before	After	Before	After	Before	After
14	6	Female	Thymic hypoplasia, nucleoside phosphorylase deficiency	68(121)[e]	214	158/93	20,601/200	134(263)/69	2,536/1,342
15a	6	"	Thymic hypoplasia	38(171)	623	7,000/350	1,081/101	631(627)/95	389/92
15b	8	"	" "	733(1,032)	ND[f]	4,610/227	ND	387/322	ND
16	34	Male	Acquired hypogammaglobulinemia	415	1,075	13,251/301	16,873/547	1,554(6,484)/100	1,276/99
17	12	Female	Cartilage hair hypoplasia	637(950)	410	4,680/307	2,330/307	1,036(1,255)/412	422/96

[a] *See* (*5*).
[b] *See* footnote *b*, table 3.
[c] *See* footnote *c*, table 3.
[d] *See* footnote *d*, table 3.
[e] *See* footnote *d*, table 3.
[f] ND = not done.

increased total numbers of T-cells. One patient's lymphocyte response to PHA and 2 patients' lymphocyte responses in MLC became normal. Each improved clinically with decreased systemic and cutaneous infections; diffuse herpes simplex of 3 years' duration resolved in 1 patient, but thrombocytopenia persisted in all of them.

A single patient with severe combined immunodeficiency disease (#11) received 5 months of thymosin therapy (table 4-6). At the age of 1 month, he received a fetal thymus transplant ip with subsequent demonstration of cell chimerism, a normal percent of T-cell rosettes, and normal T-cell function. When he was 3 years old, clinical deterioration occurred simultaneously with decreased T-cell function, which was assayed by lymphocyte response to PHA and in MLC. His T-cell rosettes remained elevated and cell chimerism, determined by HLA typing, persisted. Although lymphocyte incubation with thymosin did not enhance any cell function, a trial of thymosin was begun. After 5 months of therapy, no evidence of clinical improvement or normalization of T-cell function was observed. Thymosin was discontinued, and a cultured thymus epithelial transplant was performed. The patient subsequently died with chronic lung disease and sepsis.

Two infants with the DiGeorge syndrome were treated in this study (table 4-6), and both were diagnosed within 3 days following birth because of congenital heart disease involving the ascending aortic arch, persistent and severe hypocalcemia, and lymphopenia for age. The first infant's (#12) total T-cells were depressed, with no increase of percent of T-cell rosettes with thymosin incubation; the infant's lymphocytes responded normally to PHA but abnormally in MLC. When 10 days old, she developed a fine maculopapular rash, which was evanescent and was believed to be consistent with a GVH reaction; HLA chimerism was not present. At 20 days of age, total T-cell numbers and lymphocyte response to PHA and in MLC were depressed. Four days later, following a stormy clinical course, she died with sepsis (*Pseudomonas*) and peritonitis. The second patient (#13) with DiGeorge syndrome who received thymosin had no significant infections or evidence of a GVH reaction during his life. At the age of 3 days, his T-cell count was decreased, and his lymphocyte response to PHA and MLC was depressed. Incubation with thymosin increased both percent of T-cell rosettes and lymphocyte response in MLC. After 3 months of thymosin therapy, total numbers of T-cells and his lymphocyte response to PHA and in MLC were normal. He died at age 3 months and 1 week following cardiac surgery.

Of 2 patients with thymic hypoplasia who received thymosin therapy (table 4-7), the first, a 6-year-old female (#14) with nucleoside phosphorylase deficiency, had normal B-cell and decreased T-cell immunity. Prior to therapy, incubation with thymosin increased the percent T-cell rosettes and lymphocyte response in MLC. The total number of T-cells increased after 6 months of therapy, as did in vitro lymphocyte response to PHA and in MLC. After 6 months of therapy, the patient developed immediate hypersensitivity reactions to thymosin consisting

TABLE 4-8.—*Relationship between pretreatment of total T-cells and survival in lung cancer patients receiving thymosin*[a]

Total T-cell level	Median survival in days		Comparison between treatment groups by life-table analysis (Genan's test)
	Chemotherapy and 60 mg thymosin/m^2	Chemotherapy alone	
High: $>775/mm^3$	350 (9)	285 (11)	$P=0.28$ (1-sided)
Low: $<775/mm^3$	405 (12)	180 (8)	$P=0.006$ (1-sided)

[a] *Numbers in parentheses* = No. of patients; *see* Chretien et al. (*17*).

of urticaria at the injection sites and respiratory wheezing. Therapy was discontinued. Within 1 month, the patient's percent T-cell rosettes and lymphocyte response to PHA and in MLC decreased to pretreatment abnormal levels.

The second patient (#15) had abnormal B-cell immunity, decreased T-cells, and depressed lymphocyte response to PHA and in MLC. Incubation with thymosin enhanced T-cell rosette formation but did not alter her lymphocyte response in MLC. Therapy, begun when the child was 4 years old, was continued for 33 months. While receiving thymosin, she had conversion of her delayed hypersensitivity skin tests to mumps and *Candida* and an increase in total number of T-cells but no change in lymphocyte response to PHA or to allogeneic cells. The patient's IgG increased from 220 to 1,220 mg/dl. However, she remained unable to produce specific antibody to tetanus, pneumococcal polysaccharide, or keyhole limpet hemocyanin. She improved clinically for approximately 28 months while receiving thymosin and had decreased numbers and severity of systemic and cutaneous infections, decreased diarrhea, and weight gain (from 26 to 42 lb). Between 28 and 33 months following initiation of thymosin therapy, she deteriorated clinically with an increasing number of infections. Thymosin was discontinued. Over 6 months, she continued to deteriorate with recurrent pneumonia, otitis media, and severe skin infections and required multiple hospitalizations. Neither the total number of T-cells nor lymphocyte function decreased. However, because of clinical deterioration, thymosin was reinstituted. Following 4 months of treatment, T-cell numbers increased. Both systemic and cutaneous infections cleared, and the patient has remained clinically well.

A 34-year-old man (#16) with acquired hypogammaglobulinemia, decreased total number of T-cells, and depressed lymphocyte response to PHA and in MLC received thymosin for 18 months. Thymosin incubation did not increase T-cell rosette formation but did enhance lymphocyte response in MLC. After 6 months of treatment, the patient's total number of T-cells increased but lymphocyte response to PHA and in MLC remained depressed. Quantitative immunoglobulins and numbers of circulating B-cells were unchanged. Before therapy, the patient had recurrent infections, diarrhea, a 20-pound weight loss in 1 year, hepatomegaly, and premalignant cells on liver biopsy. After 6 months of therapy, he was clinically stable but liver morphology on repeat biopsy was unchanged.

A 12-year-old female (#17) with cartilage hair hypoplasia, normal B-cell immunity, decreased T-cells, and depressed PHA and MLC responses had received thymosin for 5 months. Before therapy, the total number of T-cells increased with thymosin incubation, although the MLC response did not change. Following treatment, no significant alteration in T-cell function was seen.

In a follow-up report of this trial of Barrett et al. (*8*) in a group of 17 patients plus another, Wara and co-workers (*7*) correlated changes of MLC reactivity in vivo following thymosin therapy with pretherapy enhancement of MLC reactivity by in vitro thymosin incubation; they found a highly positive correlation. In this retrospective analysis, pretherapy lymphocyte incubation with thymosin in vitro resulted in greater than 119% enhancement of MLC reactivity in those patients who had normal reactivity after therapy. Conversely, in those who did not develop normal MLC responses after therapy, pretherapy lymphocyte incubation with thymosin resulted in 7–9% enhancement (No. of samples = 63) of MLC reactivity. Wara et al. observed that, if the criteria for a positive enhancement in vitro are greater than 100%, 6 of 8 patients' in vitro responses correlated with subsequent response to therapy. Thus it is possible that pretherapy incubation of MLC with thymosin can be used as an indicator of the in vivo efficacy of thymosin to enhance one aspect of T-cell function. A prospective study in larger numbers of patients is necessary before we can better define "significant" percent enhancement of MLC reactivity with thymosin incubation.

In a subsequent study, Barrett et al. (*8*) observed that T-cell numbers were increased to normal with thymosin in vitro in 3 of 5 patients with DiGeorge syndrome. When thymosin was given in vivo, the same 3 patients responded by an improvement in T-cell numbers and function. Similarly, in a single case report, Sharp and Peterson (*9*) report a significant improvement of cellular immunity in a patient with the same disease who was treated with thymosin fraction 5.

In a patient with thymic hypoplasia and nucleoside phosphorylase deficiency treated for 9 months with thymosin at a dose of 1 mg/kg per week, Ammann et al. (*11*) reported an increase in the percentage of E-rosettes, the total numbers of T-cells, and a return of the PHA and MLC response to greater than 50% of normal control values. However, with the development of systemic type 2 reactions following thymosin administration, therapy was discontinued. Cell immunity returned to previous abnormal values.

In a case report by Rubenstein et al. (*12*) on a patient with adenosine deaminase deficiency and combined immunodeficiency, thymosin therapy improved the clinical course of the disease when given with transfusions of RBC. The in vivo improvement in CMI correlated with the in vitro response to thymosin. Bonagura and Pitt (*36*) studied a patient with hypothyroidism with T-cell deficiency and hypoimmunoglobulinemia and found that thymosin therapy partially corrected the T-cell defects. The patient achieved normal Ig levels after 5 months of thymosin therapy.

Phase I and II Trials with Thymosin Fraction 5

More than 200 cancer patients have been treated according to phase I (*4, 13–18*) or phase II protocols for periods up to 4 years. As with pediatric patients, no major side effects have been seen in most of them.

The first phase II randomized trial (55 patients) of thymosin has now been completed in nonresectable small cell carcinoma of the lungs by Chretien and Cohen and their associates at the Veterans Administration Medical Center, Washington, D.C. (*15–17*). As illustrated in figure 4-1, thymosin fraction 5 prolonged the survival of patients in this trial significantly when given in conjunction with intensive chemotherapy. Mean survival time was increased from 240 days with chemotherapy alone to over 450 days with chemotherapy plus 60 mg thymosin/m^2 twice per week for the first 6 weeks of the chemotherapy induction

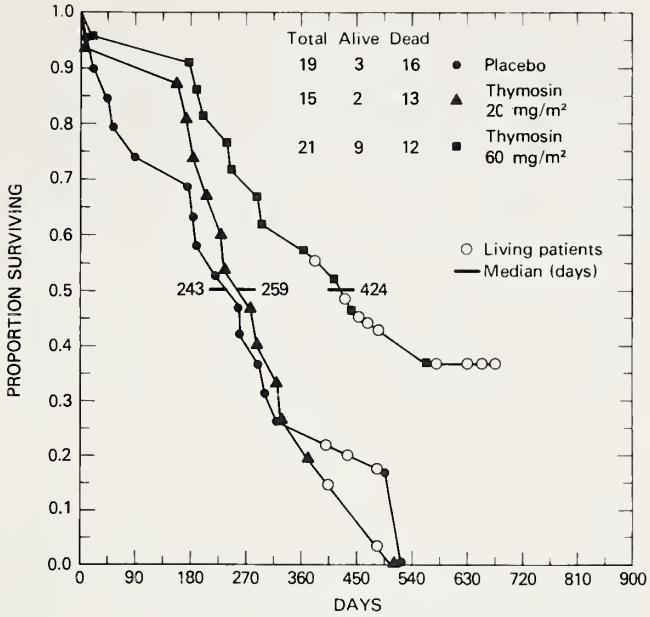

FIGURE 4-1.—Thymosin in conjunction with chemotherapy of cancer patients [*see* (*15*) and Chretien et al. (*37*)]. Survival kinetics of patients with oat cell bronchogenic carcinoma who were given 60 mg thymosin fraction 5/m^2, 20 mg thymosin fraction 5/m^2, or a placebo twice weekly sc during the first 6 wk of intensive chemotherapy. Thymosin increased median survival from 240 to over 450 days. Survival of the group receiving 60 mg is significantly greater than that of the group given the placebo ($P<0.01$), whereas those given the placebo and 20 mg did not differ significantly ($P<0.34$).

period. The median survival was over 500 days. Six of the original 21 patients in the high-dose thymosin group were alive and tumor free more than 2 years later (Cohen MH: Personal communication).

As indicated in tables 4-8 and 4-9, prolonged survival of the patients with lung cancer correlated directly with initial low levels of T-cells and low levels of α_2 HS glycoproteins (*16, 17*).

Phase I Trial With Synthetic Thymosin α_1

Using synthetic thymosin α_1 prepared by Hoffmann-LaRoche (Nutley, N.J.), Baskies and his colleagues (*38*) completed a small phase I trial in patients with brain tumors. Fourteen patients with anaplastic gliomas who had received surgical and radiation treatments with and without concurrent intratumoral 8-azaguanine therapy were entered in a phase I trial in which thymosin α_1 was tested versus thymosin fraction 5. Three patients received fraction 5 at 60 mg/m^2 sc twice weekly for 4 weeks; of those administered α_1, 4 received 900 μg/m^2, 5 were given 600 μg/m^2, and 2 received 300 μg/m^2 for the same duration. Total TL, the change in percent TL after incubation with in vitro thymosin, and routine neurological, hematological, and cardiopulmonary monitoring were done for 2 weeks before treatment, during the 4 weeks of treatment, and for 4 weeks afterward. The patients were divided into 2 groups: clinically deteriorating and clinically stable. Mean changes in percent TL after incubation were then compared for the 3 treatment groups as above. Values before and after treatment were significantly different in both groups; changes in percent TL after incubation did not appear until after the treatment was completed. In addition, serum levels of the α_2 HS glycoproteins in the serum (a measure of immune competence) were significantly increased in patients following the posttreatment. No clinical or hematological toxic effects were noted in any of the patients receiving α_1 during the trial. Two years after the start of the trial, none of the 7 patients in the deteriorating group were alive, whereas 5 of 7 patients in the thymosin-treated stable group were living. This prolonged survival of the second group is greater than would be expected with conventional therapy alone (Ommaya AK: Personal communication).

TABLE 4-9.—*Relationship between pretreatment serum α_2-HS-glycoprotein levels and survival*[a]

α_2-HS-Glycoprotein	Median survival in days		Comparison between treatment groups by life-table analysis (Genan's test)
	Chemotherapy and 60 mg thymosin/m^2	Chemotherapy alone	
High: >60.5 mg/dl	300 (10)	262 (11)	$P=0.17$ (1-sided)
Low: <60.5 mg/dl	495 (10)	180 (8)	$P=0.039$ (1-sided)

[a] *Numbers in parentheses* = No. of patients; *see* Chretien et al. (*17*).

Phase I Trial With Thymosin Fraction in Autoimmune Disease

Lavastida and Daniels (*19*) conducted a small phase I trial in patients with autoimmune diseases, 6 of whom were treated with thymosin fraction 5 for periods ranging from 4 to 16 months. Five of the patients had SLE and the sixth had rheumatoid arthritis and Sjögren's syndrome.

While the patients were on thymosin, significant changes were seen in T-cells and null cells as well as a major decrease in a cytotoxic SF present in the sera of many with autoimmune diseases. This heterologous factor causes the lysis of murine thymocytes in the presence of complement. Based on these encouraging findings, investigators have a phase II randomized trial underway at the same center to determine the efficacy of thymosin therapy in SLE (table 10).

Although the mechanism of immune reconstitution with thymosin fraction 5 in patients with rheumatoid diseases is not defined, it may be related to induction of a subpopulation of thymosin-activated suppressor or regulator T-cells.

National Cancer Institute-sponsored Clinical Trials With Thymosin

In January 1981, the NCI started additional phase I–II studies to determine proper dose, regimen, and efficacy of thymosin fraction 5 and the clinically synthesized thymosin α_1. The BRM Program is currently sponsoring 5 clinical trials.

Researchers at 5 institutions are conducting phase I–II studies on 2 forms of thymosin, thymosin fraction 5 and

TABLE 4-10.—*Changes (in percent) in lymphocyte subpopulations and decreased serum cytotoxicity following in vivo administration of thymosin to patients with autoimmune diseases*[a]

Disease	Patient No.	T-cells		Null cells		B-cells		Cytotoxicity[b]	
		Before	During	Before	During	Before	During	Before	During
Systemic lupus erythematosus	1	31	67	58	9	11	24	27	0
" " "	2	49	51–71	ND	0	ND	29	10	0
" " "	3	24[c]	71	60[c]	12	16[c]	17	76	0
Rheumatoid arthritis and Sjögren's syndrome	4	45	68	17	0	38	33	57	0
Systemic lupus erythematosus	5	37[c]	52–62	48[c]	36	15[c]	12	18	0

[a] *See* (*19*). Values given are for before and during treatment. ND = not done.

[b] Normal values = 0 to 10%. Values represent capacity of patients' sera to lyse murine thymocytes in the presence of complement. *See* methodology for details of cytotoxicity assay.

[c] Results were from early treatment.

thymosin α_1; the institutions are The Fred Hutchinson Cancer Research Center, Memorial Sloan-Kettering Cancer Center, University of California at San Diego, George Washington University, and the Northern California Cancer Program.

Thirty-five to 45 patients will be enrolled in each study, with 75 to 90 patients enrolled at centers where both substances will be tested. The notable differences are that only investigators at George Washington University will use placebo controls, and those at this University and The Fred Hutchinson Center will test only patients with non-small-cell lung cancers. In addition, scientists at George Washington will first give palliative radiation therapy and then administer thymosin to see if it will restore radiation-suppressed T-cell activity.

PRIORITY AREAS FOR DEVELOPMENT

Biochemical Characterization and Synthesis of Thymic Peptides

Considerable progress has been made in the past 2 years in the clinical characterization of several of the biologically active purified thymosin peptides, as well as with the active biological components of THF, TP II, the serum thymic factors of Bach (FTS) and White (prealbumin), and other thymic-derived or dependent factors. Given a potential role for these thymic factors in modulating immunity, a major area for priority development would be the support of research proposals for full chemical characterization of the biologically active polypeptides. These studies should include purification of material in sufficient quantities for amino acid sequence analysis. Sequence analysis of the purified polypeptides would facilitate comparison and the identification of similar or disparate biologically active components in the various thymic preparations. Major efforts should be given to the synthesis of biologically active and well-characterized thymic polypeptides so that the methodology necessary for large-scale production geared to clinical application can be developed. Classical chemical solution and solid-phase synthesis of thymosin α_1, TP II, and FTS have already been accomplished. Similar strategies should be developed with the other thymic factors.

Bioassays

One of the most difficult aspects of thymic hormone research has been the ambiguity of biological assays in the determination of effects of isolated thymic components. Increased emphasis should be placed on the development of new reproducible bioassays for the measurement of T-cell differentiation and function to supplement existing assays. These assays should include functional tests for distinguishing between helper and suppressor T-cells; identification of enzyme, antigen, and other surface markers that would help distinguish between human T-cells at various stages of differentiation and maturation; and for the development of sensitive RIA and ELISA for determining levels of circulating thymic hormones in the blood. Furthermore, scientists should develop experimental animal tumor programs which can be used to explore the efficacy of administering thymosin by various routes (locally, systemically, intratumor) and in combination with appropriate cells plus antigens (e.g., tumor antigen) and other BRM, such as lymphokines, adjuvants, and IF. Screening programs should be established that could help us determine how thymic hormone therapy in conjunction with other therapeutic modalities affects tumor growth or regression, or both. Similar screening programs should be established for the other immune BRM.

Clinical Trials with Thymosins

The initiators of confirmatory trials with thymosin fraction 5 in patients with oat cell cancer of the lung, as well as with other tumors associated with documented T-cell suppression, would have as their goal the confirmation of the findings of Chretien et al. and the exclusion of the possibility that these findings were due to factors unrelated to the administration of thymosin. Thymosin fraction 5 may contain several, if not all, of the major biologically active peptides that have been isolated from the thymus and blood. Thus if the initial phase II trials of Cohen et al. (*15*) and Chretien and associates (*17*) can be confirmed with this preparation, it would accelerate the interest in the characterization and utilization of the purified thymic peptides in cancer treatment.

Clinical assessment is needed of the efficacy of the synthetic form of a polypeptide isolated from thymosin fraction 5, i.e., thymosin α_1, which has been shown experimentally to have a 100-fold or greater effect on some immune reactivities associated with T-cell functions than that of the present compound, thymosin fraction 5. Once phase I trials are completed with thymosin α_1, researchers conducting one of the phase II trials with α_1 should include oat cell cancer of the lung in conjunction with chemotherapy to compare the efficacy of this synthetic compound with thymosin fraction 5 directly. Positive phase II trials with thymosin α_1 would offer the advantage of a substance that can be synthesized in the laboratory, and thus the technical aspects associated with the isolation of thymosin from the thymus gland of calves would be circumvented.

The clinical investigations should be conducted at institutions where the staffs have the capability of conducting immunological profile and functional studies on patients to assess the various biological effects of thymosin fraction 5 and thymosin α_1. In the completed oat cell trial, such tests appeared to identify the patients who benefited most from thymosin and, conversely, those who did not. The continuation and expansion of such studies should continue identification of these patient groups. This information offers the eventual advantage of restriction of the treatment of cancer patients with thymosin fraction 5 and thymosin α_1 to those most likely to benefit from these agents and the associated possibility of successful treatment of larger numbers of patients with a given amount of the agent.

GENERAL BACKGROUND AND REVIEW OF LITERATURE

The rapidity with which interest in the thymus hormones has expanded in the last decade is reflected by the publication of several volumes, symposia, and current reviews on the subject of the thymus and its hormones (*39–49*).

The growth of interest in the thymus and thymic hormones has resulted not only in a large number of publications but has also provided evidence that a variety of fractions and preparations from thymic tissue, blood, and other tissues that may synthesize and secrete polar and nonpolar compounds may be eligible for designation as thymic hormones. These newer data have been obtained during a period of more precise dissection of specific cell populations, the immunological responses of which may be modulated by the secretions of the endocrine thymus. In this review of the literature on thymic hormones we relied heavily on recent chapters by White (*49*) and Goldstein et al. (*50*).

CRITERIA FOR CLASSIFYING THYMIC FACTORS AS HORMONES

The most critical criterion for acceptance of any putative endocrine product as a hormone is its ability to replace specific functions of the extirpated or absent gland in experimental conditions. For the thymus, past experience indicates that a product with thymic hormone or similar activity should exhibit activity in one or more of the following biological models: 1) amelioration of immunological impairment in the neonatally thymectomized animal; adult thymectomized, immunosuppressed animal; and the nude mouse; and 2) other biological criteria, e.g., enhancement of immunological responses evaluated in various in vitro and in vivo assays reflecting the activities of T-cells and their effects on B-cells and macrophages.

These criteria do not apply to other types of immunoenhancing products that have been described as altering host immunological competence but are, on the basis of present evidence, unrelated to the role of the thymus. These include, e.g., levamisole (*51*) and the peptidoglycans (*52*), which have significant activity as assessed in selected assays reflecting immunological responsivity. However, these compounds, as well as products isolated from other organs, such as the bursa of the chicken (*53*) and the bovine parotid gland (*54*), although enhancing certain immunological parameters, like lymphocyte numbers and antibody production, are not within the scope of the defined criteria. Also, considerations of the role of the thymus and its hormones will be restricted to immunological phenomena. The interrelationships of the thymus to other endocrine glands will not be reviewed.

HISTORICAL BACKGROUND

Early contributions to the recognition of the thymus as an endocrine gland resulted from one of the classical approaches in endocrinology, i.e., the demonstration that following the removal of this gland in various species, deleterious biological consequences to the host resulted (*55*). These observations were soon followed by fulfillment of the second postulate for the establishment of an anatomical structure as an endocrine organ. This was the experimental evidence that the loss of specific functions of the thymus due to extirpation of the gland could be largely ameliorated by transplantation into the operated animal of either a thymus (*56*) or a chamber containing thymic tissue with a permeability that would not permit egress of cells but would allow passage to products produced by these cells or tissues (*57*).

The third postulate that required experimental proof of the endocrine role of the thymus was fulfilled by the evidence of several investigators that a cell-free extract of this tissue, when administered to a thymectomized animal, successfully restored the functions characteristic of the thymus gland (*58*).

A fourth postulate, the isolation and characterization of the secretory products of the thymus, was met by the demonstration by several scientists that cell-free products could be isolated and characterized chemically as substances that serve in lieu of the functions of the intact gland (*58*).

Finally, satisfaction of the last postulate necessary for the inclusion of the thymus to the list of endocrine glands has been the elucidation of the structure of several of the isolated products, their synthesis, and the demonstration that the synthetic product has some of the biological properties characteristic of the purified isolated entity (*59–62*).

In general, the history of our knowledge of the endocrine glands and their hormones has revealed that, in some instances, an endocrine gland may secrete either a single hormone or may be capable of synthesizing and secreting several hormones differing in chemical structure and in biological activities. The thymus appears to be a member of this second category of endocrine glands, inasmuch as several distinct chemical products have been prepared from thymic tissue. In some instances, these different fractions resemble one another in their biological effects; in others, the purified products mimic only certain of the established biological actions that have been attributed to the endocrine functions of the thymus gland.

In addition to the suggestive evidence that the thymus synthesizes and secretes a group of diverse hormones rather than a single entity, advances in knowledge of the functions of the end cell of thymic hormone action, i.e., the T-lymphocyte, have been accompanied by an exponential accumulation of data demonstrating the heterogeneity of lymphoid cell populations when assessed by the diverse immunological responses they are capable of manifesting. Thus the initial recognition of at least 2 major categories of lymphoid cells, T- and B-cells, has been followed by the demonstration that subsets of distinct classes of these cells can be delineated on the basis of immunological end points. This development immediately raises the question of whether a single thymic hormone can elicit all the potential biological functions of lymphoid cells, or whether several hormones are produced by the thymus, each of which may act to regulate selected, specific immunological roles of lymphoid cells. This last possibility would not only explain the diverse nature of the products that have been reported with thymus hormone or thymus hormone-like activity but may also have some significance for the future of more precise practical application of these substances for the amelioration of various types of impaired immunological states in a number of clinical conditions.

In the pages that follow, consideration is given to the chemistry of 1) purified factors isolated from thymic tissues, and 2) purified factors with thymic hormone-like activity isolated from blood. Subsequently, the biological and biochemical effects of the isolated products will be summarized. The designation of a product as a thymic hormone logically connotes that the isolated material is obtained from the thymus and exhibits biological effects established as characteristic of this gland. Alternatively, a preparation with thymic hormone-like activity might be present in nonthymic tissue, e.g., the blood, either as a de novo secretory product from the thymus or as a fraction derived from chemical alterations that have occurred in the gland of origin or subsequent to entrance into the circulation. In this last situation, in the absence of evidence that the putative hormone can be identified as a constituent of the thymus, its biological actions should at least resemble those of extracts of the gland itself.

It is on the basis of the foregoing that a selection has been made from the literature of the various products that satisfy the above criteria and have been either partially or completely characterized chemically.

CHEMISTRY AND BIOLOGY OF HORMONES AND FACTORS PREPARED FROM THYMIC TISSUE

Thymosin and Its Composite Polypeptides

Thymosin Fractions 5 and 5A

Purification.—As outlined in figure 4-2, thymosin fraction 5 is prepared from calf thymus, as described by Hooper et al. (*63*). The thymus tissue is homogenized and centrifuged, and the supernatant is filtered through glass wool. The filtrate is then processed through 3 steps: 80° C heat, acetone precipitation, and ammonium sulfate precipitation. The 25–50% ammonium sulfate precipitate is further subjected to ultrafiltration in an Amicon DC-2 hollow fiber system and desalted on a Sephadex G-25 column to yield fraction 5. The 50–95% ammonium sulfate cut is also collected and processed through DC-2 and Sephadex G-25 to yield fraction 5A.

Nomenclature of thymosin polypeptides.—Analytical isoelectric focusing of thymosin fraction 5 has revealed the presence of a number of components in the preparations. A nomenclature based on the isoelectric focusing pattern of thymosin fraction 5 and the pH range of 3.5–9.5 has been described (*59*) and is illustrated in figure 4-3. The separated polypeptides are divided into 3 regions: The α region consists of polypeptides with isoelectric points below 5.0, the β region 5.0–7.0, and the γ region above 7.0. The subscript numbers α_1, α_2, β_1, β_2, etc. are used for identification of the polypeptides from that region as they are isolated. The purified polypeptides are tested in various assay systems in investigations of their biological efficacy.

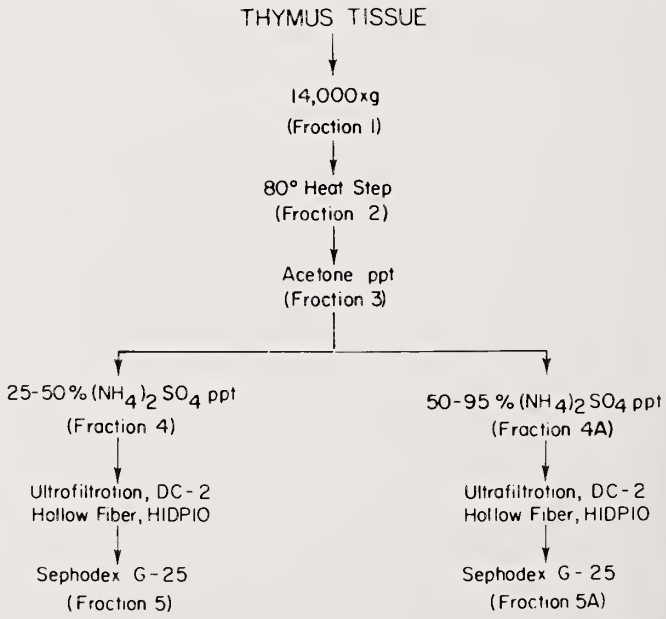

FIGURE 4-2.—Purification of bovine thymosin fractions 5 and 5A. One kilogram thymus tissue homogenized in 3 liters of NaCl solution provides the sample for one to initiate the procedure (*64*). HIDPIO = the model number of the Amicon BC2 hollow fiber system used.

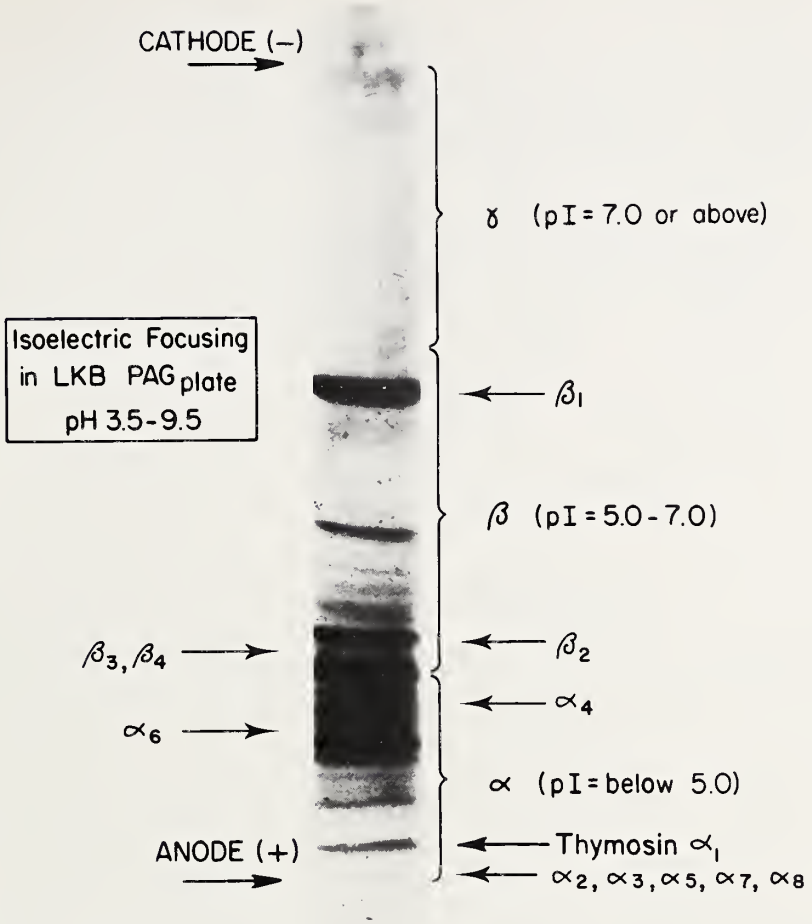

FIGURE 4-3.—Thymosin fraction 5 contains a family of small acidic polypeptides with mol wt ranging from 1,000 to 15,000 (*59*).

The active polypeptides are then given the prefix "thymosin;" the components which are inactive in bioassay systems and are believed not to be involved specifically in controlling T-cell maturation and function are given the prefix "polypeptide."

Purified thymosin polypeptides.—The major effort of scientists in the thymosin research program has been to understand the molecular events by which the thymus gland exerts control over T-cell development. To understand the detailed mechanism(s) of how each thymosin polypeptide exerts control over the development and senescence of immunological responses, one must isolate and characterize each polypeptide component in thymosin fraction 5 and determine the individual biological functions.

Ongoing studies suggest that some of these peptides may act at different sites and on different subsets of T-cells and contribute to the maintenance of immune function and balance (fig. 4-4).

Bovine Thymosin α_1

The first thymosin polypeptide isolated from the highly acidic region of fraction 5 has been termed thymosin α_1. This peptide is highly active in several bioassay systems (table 4-11). Thymosin α_1 was isolated from fraction 5 by ion-exchange chromatography on CMC and DEAE cellulose and by gel filtration on Sephadex G-75 in guanidine hydrochloride (*59, 64, 66*). The yield of thymosin α_1 from fraction 5 is about 0.6%. Thymosin α_1 is a polypeptide consisting of 28 amino acid residues with a mol wt of 3,108. The complete amino acid sequence (*59, 66*) of this peptide is shown in figure 4-5; its amino terminus is blocked by an acetyl group.

Comparison of the sequence of thymosin α_1 with the published sequence of other factors (*see* fig. 4-5), such as TP II (*67*) and serum thymic factor (*68*), reveals no homology. Computer analysis of the sequence of α_1 has established that α_1 bears little homology to any of the 957 protein sequences that have been published to date (*59*).

Recently, Freire and co-workers (*69*) at the Roche Institute (Nutley, N.J.) performed experiments to determine whether some of the reported thymic hormones are actually synthesized in the thymus gland. The translation of mRNA isolated from calf thymus was done in the cell-free wheat germ system. The radioactive products that were immunoprecipitable with antisera against thymosin fractions were analyzed and found identical to those expected for tryptic peptides from thymosin α_1. More recently, Freire et al. (*70*) purified the mRNA fraction from calf thymus polysomes by preparative PAGE.

The results of these studies suggest that thymosin α_1 is synthesized in the thymus as a longer peptide chain of 16,000 daltons and may be further processed (or degraded)

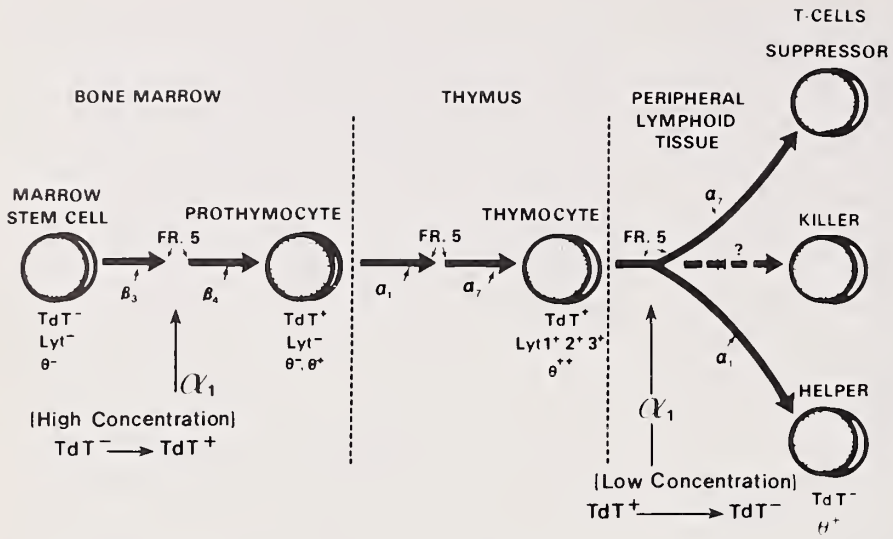

FIGURE 4-4.—Proposed role of thymosin peptides in T-cell maturation. *See* (*65*).

TABLE 4-11.—*Summary of thymosin activity in various bioassays*[a]

Assay	Thymosin fraction 5, μg	Thymosin α_1, μg
MLR	1–10	
MIF	1–5	0.001–0.1
E-rosette	1–10	0.001–0.01
Mitogen	1–10	0.01 –0.1
Antibody	10–100	NA
$Lyt^{1+,2+,3+}$	10–100	0.01 –0.05
TdT	0.01–0.01,[b] 100[c]	NA[b], 1[c]

[a] *See* (*47*). Ubiquitin was not active. NA = not active.

[b] Value indicates induction of TdT in BSA-separated bone marrow cells in vitro from nu/nu mice.

[c] Value indicates induction of TdT in vivo in thymocytes from HCA-suppressed C57BL/6J mice.

to form the peptide detected in preparations isolated from the thymus.

Wetzel and associates (*71*) reported the isolation and complete chemical characterization of a N^{α}-desacetyl thymosin α_1 using recombinant DNA procedures. In this important new development, the gene for thymosin α_1 (fig. 4-6) was synthesized, inserted into a plasmid, and cloned in a strain of *E. coli*. The primary nucleotide sequence required for the gene was accomplished with the use of prepared prokaryotic codons with elimination of codons of multiple specificities. In the cloning of the α_1 gene, the gene was inserted under *lac* operon control into the plasmid and expressed as part of a β-galactosidase chimeric protein. The structure of the N^{α}-desacetyl thymosin α_1 was confirmed by sequence analysis and, as indicated in tables 4-11 and 12, the molecule was as

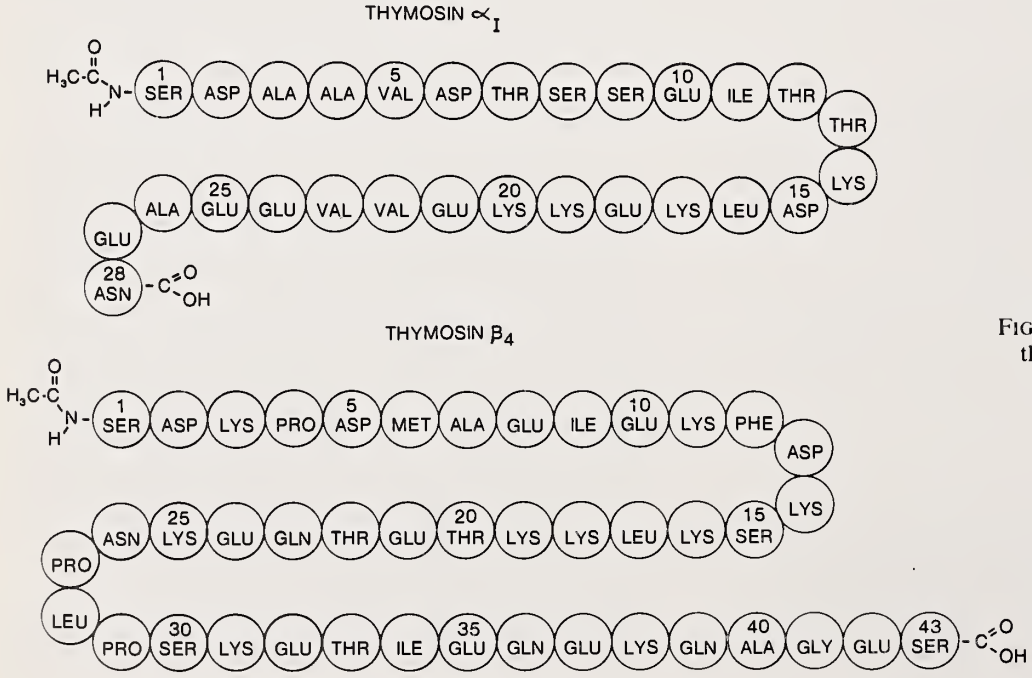

FIGURE 4-5.—Sequence analysis of thymosin α_1 and β_4.

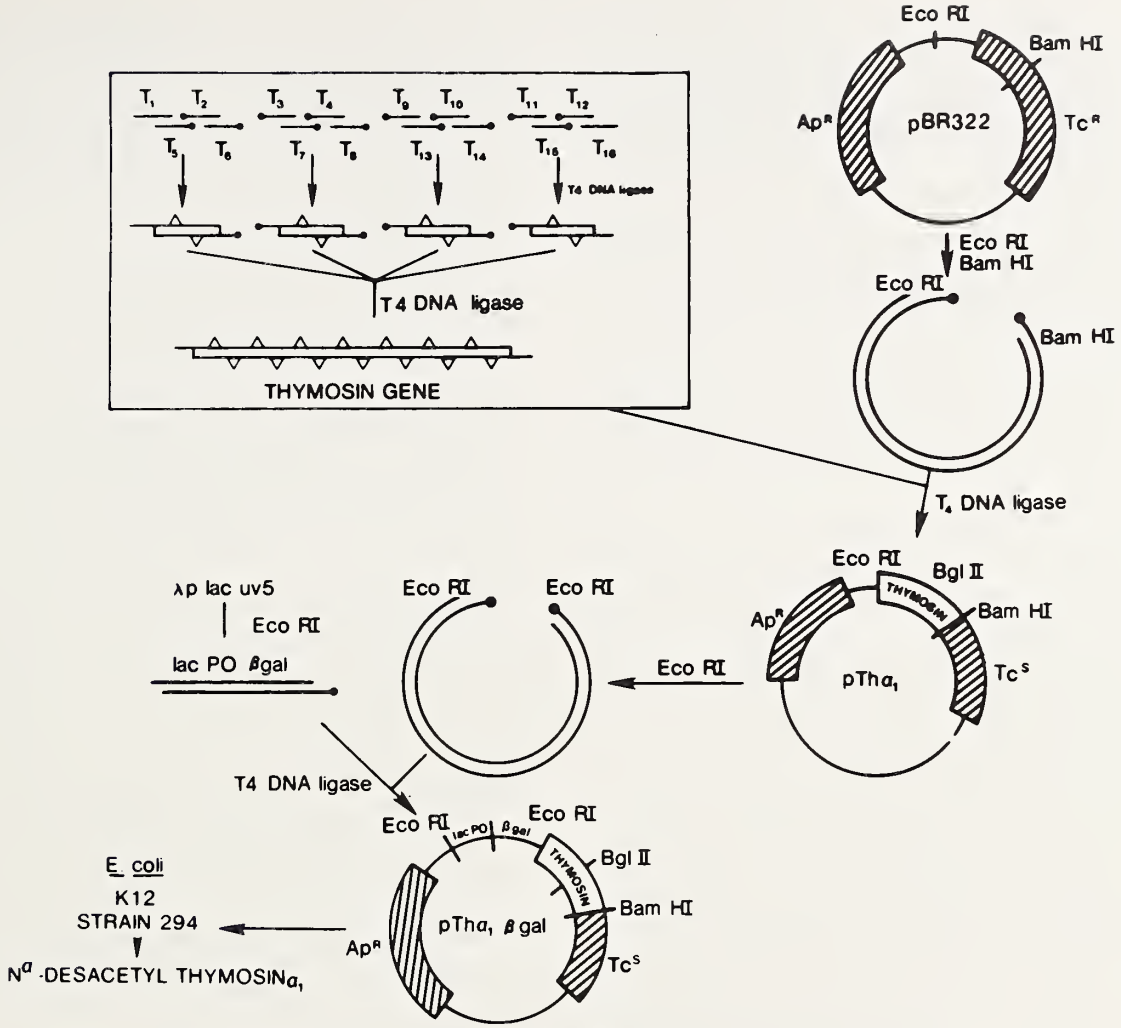

FIGURE 4-6.—Construction of gene and plasmid for N^{α}-desacetyl thymosin α_1.

biologically active in vitro as the chemically synthesized thymosin α_1. A method for aminoacetylation of the N^{α}-desacetyl thymosin α_1 has been reported by Kido et al. (*72*) who used a transacetylase associated with a wheat germ ribosomal preparation.

Synthetic Thymosin α_1

Thymosin α_1 has been chemically synthesized by Wang and co-workers (*73*) (according to the solution method) using *t*-butyloxy-carbonyl-amino acids as building blocks. The purified material migrates as a single spot on acrylamide gel isoelectric focusing (pH 3.5 and 9.5) and on high-voltage silica gel thin-layer electrophoresis (pH 1.9 and 5.6), indistinguishable from the natural thymosin α_1. The synthetic polypeptide migrates to the same position as the natural α_1 preparation on LKB PAGE plate with a pH range of 3.5–9.5 (fig. 4-7). The synthetic α_1 was tested in MIF and E-rosette assays and appears to have activity similar to the natural material. A summary of the biological activities of thymosin α_1 is given in table 4-12.

Thymosin α_1 From Other Species

To evaluate the species variation of the thymosin polypeptides, Low and Goldstein (*47*) prepared thymosin fraction 5 from thymus tissues of different species, including the human, pig, sheep, chinchilla, and mouse. The human thymus tissue was excised during open heart surgery and from selected autopsies. Thymosin α_1 from several animal species was prepared from fraction 5 by a modification of the extraction and fractionation procedures developed for the isolation of bovine α_1. Human, porcine, and ovine thymosin α_1, also partially sequenced (Low TK, Hu SK, Goldstein AL: Unpublished observations), appear to have an identical sequence to bovine α_1.

Thymosin α_5 and α_7

Both of these partially purified peptides were isolated from fraction 5 by ion-exchange chromatography on CMC and DEAE cellulose and gel filtration on Sephadex G-75 (Low TK, Goldstein AL: Unpublished observations). They are highly acidic with isoelectric points around 3.5 and are

TABLE 4-12.—*Some biological properties of thymosin and its component polypeptides*[a]

In vitro enhancement of:
- Number of azathioprine-sensitive, E-rosette-forming spleen cells from adult, thymectomized mice
- Appearance of phenotypic T-cell markers on mouse spleen and bone marrow cells
- Responsivity to mitogens
- MLR
- Conversion of bone marrow cells into cells reactive in the GVH reaction in vivo
- Production of suppressor T-cells, killer T-cells, and MIF
- Production of antibody to SRBC by spleen cells of normal and thymectomized mice
- Intracellular cGMP levels of mouse spleen cells
- Specific activity of TdT of normal mouse thymocytes (suppression of induction of the enzyme observed above optimum concentrations of thymosin fraction 5 and thymosin α_1)
- Ecto-5′-nucleotidase activity in human thymocytes

In vivo enhancement of:
- Lymphocytopoiesis in normal, germfree, adrenalectomized, neonatally thymectomized, and athymic mice
- Rate of allograft rejection in normal and neonatally thymectomized mice
- Resistance of progressive growth of Moloney virus-induced sarcoma in normal mice
- MLR (in vivo–in vitro) by lymphoid cells from normal or neonatally thymectomized mice
- Lymphoid cell response to mitogens (in vivo–in vitro) by cells of normal and athymic mice
- Resistance to growth of allogeneic and xenogeneic tumors in athymic mice
- Delay of abnormal thymocyte differentiation (loss of suppressor function) in NZB mice
- Antibody production to SRBC (in vivo + in vitro)
- Survival in tumor-bearing mice (in conjunction with chemotherapy)
- IF production following viral challenge

Thymosin α_1

In vitro enhancement of:
- Numbers of E-rosette-forming cells in cancer patients
- Autologous rosette-forming cells in cancer patients
- Secondary T-cell-dependent IgG, IgM, and IgA antibody response
- MIF and helper T-cells
- Expression of Ly-1,2,3 positive cells
- T-cell-dependent specific antibody production

In vitro suppression of:
- TdT activity in murine thymocytes
- Elevated Tγ:Tμ ratios in peripheral blood of cancer patients

In vivo enhancement of:
- Lymphoid cell responses to mitogen (in vivo + in vitro)
- Lymphotoxin production (in vivo + in vitro)
- Survival in tumor-bearing mice (in vivo + in vitro) and in conjunction with chemotherapy
- IF production following viral challenge

Thymosin α_7
- In vitro enhancement of suppressor T-cells and Ly-1,2,3 positive cells
- In vivo enhancement of suppression of IF production following viral challenge

Thymosin β_3 and β_4

In vitro enhancement of:
- Induction of TdT in separated bone marrow cells from normal or genetically athymic mice
- MIF-like activity in guinea pig and murine macrophages
- Macrophage tumoricidal activity

In vivo enhancement of:
- Induction of TdT in thymocytes of steroid suppressed mice
- Induction of TdT in separated bone marrow cells from normal or genetically athymic mice

[a] *See (46–48, 58, 73).*

free of carbohydrate and lipid. Thymosin α_5 has a mol wt of 3,000 and thymosin α_7 of 2,200.

Polypeptide β_1

The most predominant band on isoelectric focusing of thymosin fraction 5 is polypeptide β_1 (fig. 4-3). This peptide was isolated from fraction 5A by chromatography on DEAE cellulose and gel filtration on Sephadex-75 (*64*). The amino acid sequence of β_1, composed of 74 amino acid residues, has a mol wt of 8,451 and an isoelectric point of 6.7. We believe that this peptide is not involved in thymic hormone action based on our observation that it was not active in any of our assay systems. The sequence of β_1 was identical to ubiquitin (*74*) and a portion of protein A24, a nuclear chromosomal protein (*75*). Hunt and Dayhoff (*76*) postulated that ubiquitin is a degradative product of A24.

Thymosin β_3 and β_4

These partially purified preparations, isolated from fraction 5A by chromatography on DEAE cellulose and gel infiltration on Sephadex G-75, induced terminal deoxynucleotidyl transferase positive cells. Thymosin β_3 has an isoelectric point of 5.2 and a mol wt of approximately 5,500; thymosin β_4 has an isoelectric point of 5.1 and a mol wt of 4,982. They appear to share an identical sequence through most of their amino terminal part and differ in the carboxyl terminal ends. The recently completed sequence of β_4 (*77*) is shown in figure 4-6.

Review of Biological Studies With Thymosin

These thymosin studies, initiated at the Albert Einstein College of Medicine in New York by White and Goldstein, contributed to the elucidation of the endocrine function of the thymus gland in the development and maintenance of the immune system (*63–66, 78–88*). Thymosin fraction 5 was the first well-defined thymic hormone preparation to be used clinically in patients with primary immunodeficiency diseases (*1–12*), in advanced cancers (*13–18*), and in autoimmune diseases (*19*). Its biological properties and component polypeptides are given in table 12.

Studies in Animals

Thymosin preparations have been effective in partially or fully inducing and maintaining immune function in

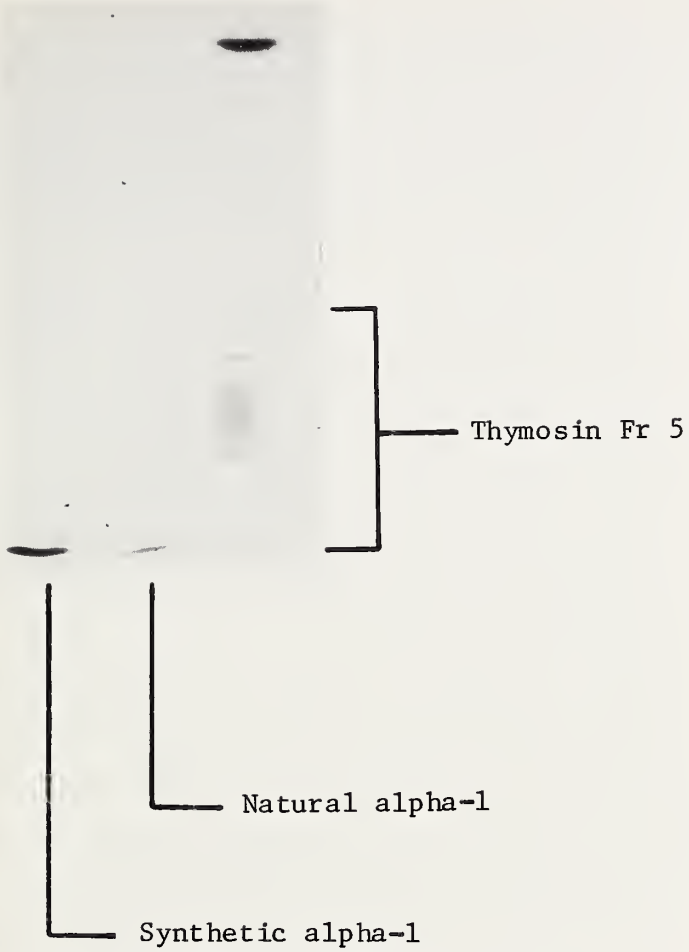

FIGURE 4-7.—Isoelectric focusing gel of synthetic and natural thymosin α_1. *See* (*59*).

various normal and immunodeficient animal models (*63–66, 78–109*). Thymosin treatment increased the survival of neonatally thymectomized mice (*86, 87*), accelerated skin graft rejection (*83, 84*), and restored or accelerated GVH reactions (*82*) and the development of immune functions in newborn mice (*86, 94, 95*). Thymosin affects the responsivity of lymphocytes from nude (*98, 99, 101, 107*), normal (*89, 99, 106*), and tumor-bearing mice and rats (*94, 95, 110, 111*) and causes immature murine lymphoid cells to acquire distinctive T-cell surface antigens (*88, 105, 106, 112, 113*). In recent experimental approaches, thymosin has shown that it is effective in inducing specific types of lymphocytes, i.e., killer, helper, and suppressor cells (*100–102, 109, 113*), and that specific purified thymosin peptides isolated from thymosin fraction 5 can induce certain markers (TdT, Thy-1, and Lyt), functional expressions of lymphocyte maturation (*88, 93, 105, 107*), and enhancement of production of a number of lymphokines, such as MIF (*114*) and IF (*115*). Most recently, thymosin fraction 5 at the optimal doses significantly prolonged survival in BALB/c mice with syngeneic plasmacytomas (MOPC-315) when administered with chemotherapy (CPP). This animal model should be most valuable in screening other thymic factors for antitumor properties (*116*).

Several provocative reports point to a role of thymosin in the aging process and in the treatment of autoimmune disease. These studies have shown that: 1) Thymosin treatment of NZB mice can reconstitute suppressor cell and other T-cell functions and temporarily cause remission of some of the symptoms of autoimmune disease commonly seen in these mice, including induction of suppressor T-cells and restoration of antigen-induced depression of DNA synthesis (*100–102*). 2) Administration of thymosin significantly increases the hemagglutinin response of aged mice (*103*), but when given to casein-treated mice, it reduces the incidence of amyloidosis (*104*). 3) In vitro administration of thymosin to aging thymectomized rats shortens the median $S + G_2$ phases of the spleen cell cycle to the levels seen in young rats (*108*).

Terminal Deoxynucleotidyl Transferase Assays for Thymosin

In vitro and in vivo TdT assays have been reported for the evaluation of thymosin activity in bone marrow from normal athymic mice (*93*). In another in vivo model, Hu et al. (*117*) found that thymosin fraction 5 and a purified peptide isolated from fraction 5 termed thymosin β_3, when administered to HCA-treated mice, significantly accelerates the reappearance of TdT^+ thymocytes. Moreover, they determined that low concentrations of thymosin fraction 5 induce, whereas high levels suppress, induction; it has been demonstrated further that the inducing peptides are thymosin β_3 and β_4, both of which act before the prothymocyte stage.

Also, thymosin α_1, a potent inducer of helper T-cells, was extremely effective in turning off TdT^+ thymocytes. Thus chemically distinct peptides were isolated from thymosin fraction 5 that have the capacity to induce early and late events in the differentiation process of T-cells (*see* fig. 4-4). Different doses of thymosin fraction 5 and other purified thymosin polypeptides are injected into HCA-treated C57BL/6J mice daily for 9 to 11 days. The animals are killed, thymocytes prepared, and TdT activity determined according to the method described by Pazmino et al. (*118*).

Studies in Humans

Incubation of PBL with thymosin in vitro increases with percent of total E-rosette levels of aged normal individuals and a significant number of patients with primary immunodeficiencies (*1–12*), cancer (*13–18, 119*), allergies (*120*), severe burns (*121*), leprosy (Thurman GB, Goldstein AL: Unpublished observations), viral infections (*122*), SLE, and rheumatoid arthritis (*123, 124*).

This E-rosette assay is a useful diagnostic tool in identifying patients who may respond to thymosin therapy in vivo based on their capacity to respond to thymosin in vitro. Thymosin increases both the percent and absolute number of E-rosettes formed in vitro by PBL obtained from patients with many types of thymic-dependent immunodeficiency diseases and cancer. An example of the correlation between in vitro response and in vivo increase in PBL is shown in table 4-13. Thymosin does not increase

TABLE 4-13.—*E-Rosettes in 27 cancer patients*[a]

Prethymosin (in vitro)		Postthymosin (in vitro)	
E-rosettes, %		E-rosettes, %	
Initial	Responders[b]	Initial	Responders[c]
<45	80	<45	89[d]
>45	12	>45	47

[a] *See* (*3*).

[b] Peripheral blood E-rosette-forming cells are significantly increased after incubation with thymosin in vitro.

[c] Peripheral blood E-rosette-forming cells are significantly increased following thymosin injection.

[d] One patient was evaluated but not treated with thymosin.

E-rosettes of most normal individuals with initial T-cell levels of greater than 50%.

Patients with severe combined immunodeficiency (presumably lacking a lymphoid stem cell population) do not respond to thymosin in vitro (*1*). Patients with active SLE respond significantly to thymosin in vitro, whereas individuals with the inactive type are not responsive (*124*). Individuals with a history of allergy respond to thymosin in vitro (*120*).

Cohen et al. (*125*) reported that thymosin fraction 5 or thymic epithelium-conditioned media, when incubated with human thymocytes, significantly increased ecto 5′-nucleotidase activity. Their research suggests that expression of T-cell 5′-nucleotidase activity serves as a marker of T-cell differentiation.

In a study of 388 patients with head and neck, mediastinal, and pelvic cancers who received radiation therapy and of 277 normal adults, the in vitro positive response to thymosin correlated with radiation portal for the cancer patients and with initial T-cell levels for both groups (*119*).

Incubation with thymosin in vitro induced the appearance of suppressor T-cells in the PBL of patients with both active and inactive SLE as measured in an MLR assay (*126*).

Incubation with thymosin of a subpopulation of null cells isolated by BSA density gradient from normal adult controls resulted in the induction of T-cell rosettes. These same cells enhanced responsiveness to PHA and increased MLC capacities (*127*). Within the null cell compartment after incubation with thymosin fraction 5, no changes in B-cells, monocytes, Fc-positive, or C3 receptor-bearing cells could be documented. Therefore, in normal adult controls, putative stem cells can be induced to form T-cell rosettes following incubation with thymosin fraction 5.

Cells contained within the null cell $HTLA^+$ compartment enriched by sequential nylon column filtration and E-rosette depletion could be induced by thymosin fraction 5 incubation in vitro to form T-cell rosettes; $HBLA^+$ cells were not altered by thymosin incubation (*128*). Thus a subpopulation of null cells isolated from *normal* human PBL can be induced to form T-cell rosettes by incubation with thymosin fraction 5.

Effect of Thymosin α_1 In Vitro on Peripheral Blood T-cell Rosettes and $T\gamma$:$T\mu$ Lymphocytes From Patients and Normal Individuals

E-rosettes.—The effect of short-term incubation of thymosin α_1 in vitro on T-cell rosettes, studied by Thurman et al. (*129*), was documented by the finding that thymosin α_1 is up to 100 times as active as fraction 5 in increasing the percentage and absolute number of T-cell rosettes.

Autologous E-rosettes.—A subpopulation of human T-cells representing approximately 26% of the lymphocyte pool of healthy individuals can bind autologous RBC (*130*). However, in cancer patients, this subpopulation of T-cells is significantly reduced (*131*). Caroux et al. (*132*) showed that short-term incubation with thymosin α_1 produces a significant and constant increase in autologous RBC to normal levels in patients with solid tumors.

$T\gamma$:$T\mu$ lymphocyte levels.—Chretien and associates (Personal communication) analyzed the effects of short-term incubation in vitro of thymosin α_1 on the ratio of $T\gamma$ (suppressor):$T\mu$ (helper) lymphocyte levels in normal individuals and in cancer patients. Thymosin α_1 had no effect on $T\gamma$:$T\mu$ in normal individuals; the ratios remained less than 1. However, the number of suppressor T-cells, elevated in cancer patients, resulted in elevated $T\gamma$:$T\mu$ ratios. Short-term incubation with thymosin α_1 had a highly significant effect in lowering these ratios to normal levels. The results of this study suggest that thymosin α_1 plays an important role in regulating the ratio of suppressor to helper T-cells in the peripheral blood and is thereby effective in restoring the immune balance in cancer patients with elevated suppressor T-cells.

Thymic Humoral Factor

Chemistry

The interests of Trainin and his associates at the Weizmann Institute of Science in investigating cell-free thymic extracts and their capacity to improve immunological competence stems in part from the early observations made by Trainin when he worked in Law's laboratory at the NCI (*133, 134*). They demonstrated that when thymus tissue in a Millipore chamber was placed in neonatally thymectomized mice, the specific immunological competence in these animals was restored. Subsequently, Trainin and his colleagues pursued these observations by preparing cell-free extracts that conferred in vitro immune competence to spleen cells from neonatally thymectomized mice. In the initial bioassay, an in vitro model of the GVH reaction, the investigators reported it was possible to assess the immunocompetence of isolated lymphoid cell populations to induce in vitro an increase in weight of an allogeneic spleen explant, whereas spleen cells from neonatally thymectomized mice did not achieve this competence unless they were previously incubated with thymic extracts.

The initial product was usually obtained from calf thymus. However, as is true with other investigations in this area, mouse syngeneic extracts were also active in the above assay.

The method used for the isolation of purified THF has as an initial step essentially that as described from our laboratory. However, subsequent purification procedures do not involve the heat step for the removal of inactive proteins but utilize the fact that the active product in the crude extracts apparently was of small molecular weight and would therefore be in the dialysate when these extracts were subjected to prolonged dialysis against distilled water for 60 hours in the cold. The dialysate was lyophilized, and this relatively crude fraction was used in the examination of its biological activity in various assays. More recently, further purification of THF to homogeneity has been achieved (*134*). The procedure is as follows: The dialysate was subjected to successive chromatographic steps on Sephadex G-10 and G-25 and DEAE Sephadex A-25. The product, eluted by 0.15 *M* NaCl from this last column with a linear gradient of salt, was desalted on a Sephadex G-10 column, concentrated by lyophilization, and its homogeneity was established by isoelectric focusing on polyacrylamide gels. The isoelectric point was 5.6. The amino acid composition of the purified THF is shown in table 4-14. With leucine as unity, the minimal mol wt is 3,220.

No relationship is apparent between the composition of THF and thymosin α_1. Data for the amino acid sequence of THF are awaited with interest. However, the data available permit the conclusion that the THF molecule is different from both the original calf thymosin fraction 8 and thymosin α_1. Publication of further data from Goldstein and Trainin and their associates should aid in the establishment of whether THF is a component of the family of polypeptides described in calf thymosin fraction 5. Folkers et al. (*135*) suggested that the peptide in the THF responsible for the cAMP elevating activity may be glutathione.

Biology

Until recently, the extensive in vitro and in vivo biological data reported for THF were obtained with a crude fraction prepared from calf thymus. Although certain of the extensive biological data obtained with the earlier crude calf thymic fractions (table 4-15) have not been reassessed with the homogeneous THF, certain of these assays have been repeated with the homogeneous product (*136*).

TABLE 4-14.—*Amino acid composition of THF*[a]

Amino acid residue	No. of residues (leucine = 1)
Aspartic acid	4
Threonine	1
Serine	5
Glutamic acid	8
Proline	2
Glycine	5
Alanine	2
Leucine	1
Lysine	1
Arginine	2

[a] *See* (*133*).

TABLE 4-15.—*Some biological properties of THF*[a]

Restoration of the ability of spleen cells from neonatally thymectomized mice and incubated with THF in vitro to participate in an in vitro GVH reaction
Increase of the in vitro survival of thymocytes incubated in the presence of HCA
Enhancement of the ability of normal spleen cells to respond to PHA and Con A
Enhancement of elicitation of an MLR by spleen cells from neonatally thymectomized mice incubated in vitro with THF or by spleen cells from neonatally thymectomized mice treated in vivo with THF
Restoration of the ability of neonatally thymectomized mice to reject to allogeneic skin graft or an inoculum of allogeneic fibrosarcoma cells
Restoration of ability of spleen cells from neonatally thymectomized donors to induce in vivo a GVH reaction
Restoration of capacity of T-cells from thymectomized donors to differentiate into cytotoxic cells
Promotion of T-cell helper function in thymus-deprived mice
Inhibition of generation of effector cells in vitro during the sensitization phase as measured by cell-mediated cytotoxicity
Enhancement in vitro of the synthesis of cAMP by mouse thymocytes
Enhancement of the intracellular spleen cell concentration of cAMP of spleen cells from neonatally thymectomized mice treated in vivo with THF
Enhancement of T-cell rosettes

[a] *See* (*41, 57, 134, 136*).

Thymopoietin

First isolated by G. Goldstein (*137–139*), TP was initially named thymin. The preparation of this peptide resulted from experimental studies related to the human disease myasthenia gravis, which is characterized by a deficit in neuromuscular transmission and thymic malfunction. Based on the impairment of neuromuscular transmission with thymin (TP), the neuromuscular blocking substance TP was isolated from thymus tissue (*139*). It can also induce differentiation of bone marrow cells to T-cells in vitro and hence has been claimed to be a putative thymic hormone. The purification procedures include homogenization, a heat step (70° C, 30 min), and filtration procedures through gauze and cotton. After concentration on Diaflo (XM100A; UM2) and two passages on Sephadex G-50, the eluates (twice the void volume) are lyophilized and fractionated on hydroxyapatite. The active volumes are desalted (G-25) and provide 2 peaks of activity when eluted from QAE Sephadex; the yield is approximately 1 mg/kg of tissue. The 2 isopeptides TP I and II are apparently related to peptide mapping and immunological cross-reactions; TP II has a mol wt of 5,562 and an isoelectric point of 5.5. The sequence of the molecule has been delineated and is presented in figure 4-5 (*140*).

G. Goldstein (*123*) changed the nomenclature of his preparations to avoid confusion with the pyrimidine base thymine and designated his products as TP I and II, in view of their apparent capacity to stimulate the maturation of lymphoid tissue. The 2 preparations appeared to be closely related polypeptides because they were immunologically

cross-reactive and had indistinguishable biological activities. This close relationship was confirmed when it was shown that TP I and II differ by only 2 residues (*140*). Inasmuch as these substitutions do not affect the biological activity of TP, Goldstein (*138*) suggested that the 2 preparations probably represent isohormonal variations in cattle.

With the burgeoning interest in the role of cell-free thymic extracts in immunological assays, Goldstein and his colleagues initiated studies to determine whether their factors might induce the expression of various T-cell antigens in normal bone marrow cells. This research led to further purification studies of extracts from bovine thymus. A third polypeptide, termed "ubiquitin" because of its wide distribution in nature in cells other than the thymus, was also isolated. The amino acid sequences of all of these molecules have been established (*74, 140*). The wide occurrence of this polypeptide was supported by the demonstration that ubiquitin isolated from human and bovine thymus had identical amino acid sequences. The sequences of TP I and II and ubiquitin are shown in figure 4-8. Recently, Fujino et al. (*141*) achieved a classical solution synthesis of the entire 49-amino acid chain of TP II and established that the product had biological activity similar to native TP II.

A tridecapeptide fragment of TP corresponding to residues 29 through 41 was synthesized by solid-phase methodology and shown to have the biological activity of 3% of the parental activity of the entire molecule (*61*), i.e., it exhibited selectivity for T-cell differentiation in vitro. A synthetic hexadecapeptide, representing the C-terminal portion of ubiquitin, mimics the actions of the native molecule in induction of T- and B-cell differentiation in vitro. In addition, a pentapeptide (arginine-lysine-asparagine-valine-tyrosine) corresponding to residues 32 to 36 of the 49-amino acid sequence of TP (*61*) has also been synthesized.

Comparisons of the data from G. Goldstein and those of A. L. Goldstein and their colleagues, as well as the amino acid analysis of Trainin's THF, suggest that TP I and II are distinct thymic factors. At the time this section is being written, no evidence of the identity of these thymic factors with any other structures of the polypeptides in calf thymosin fraction 5 has been published. However, as described previously, the sequence of ubiquitin appears to be identical with that of a peptide isolated from thymosin fraction 5 by Low et al. (*64, 66*) and termed β_1 peptide. The β_1 peptide and ubiquitin are the N-terminal 74 amino acids of A24, a nonhistone chromosomal protein (*75, 142*).

In view of the close similarities in chemical properties of TP I and II, the biological data reported for these polypeptides have generally been obtained with TP I. Some of these data are presented in table 4-16.

In the late seventies, Weksler and co-workers (*143*)

THYMOPOIETINS

```
                                        5                   10                  15
Thymopoietin I   NH2-Gly-Gln-Phe-Leu-Glu-Asp-Pro-Ser-Val-Leu-Thr-Lys-Glu-Lys-Leu-
Thymopoietin II     -Ser-

                                  20                  25                  30
                -Lys-Ser-Glu-Leu-Val-Ala-Asn-Asn-Val-Thr-Leu-Pro-Ala-Gly-Glu-Gln-

                             35                  40                  45
                -Arg-Lys-Asp-Val-Tyr-Val-Gln-Leu-Tyr-Leu-Gln-His-Leu-Thr-Ala-Val-
                                                                    -Thr-

                     49
                -Lys-Arg-COOH
```

UBIQUITIN

```
    1               5                  10                  15                  20
NH2-Met-Gln-Ile-Phe-Val-Lys-Thr-Leu-Thr-Gly-Lys-Thr-Ile-Thr-Leu-Glu-Val-Glu-Pro-Ser-Asp-

              25                  30                  35                  40
-Thr-Ile-Glu-Asn-Val-Lys-Ala-Lys-Ile-Glu-Asp-Lys-Glu-Gly-Ile-Pro-Pro-Asp-Gln-Gln-Arg-Leu-

     45                  50                  55                  60                  65
-Ile-Phe-Ala-Gly-Lys-Gln-Leu-Glu-Asp-Gly-Arg-Thr-Leu-Ser-Asp-Tyr-Asn-Ile-Gln-Lys-Glu-Ser-

                70              74
Thr-Leu-His-Leu-Val-Leu-Arg-Leu-Arg-COOH
```

FIGURE 4-8.—Sequence of TP and ubiquitin.

TABLE 4-16.—*Some biological properties of TP*

In vitro activities:
Enhancement or increase in:
Induction of differentiation of prothymocytes to thymocytes, as detected by cell surface markers and functional characteristics
Intracellular cAMP concentration in thymocytes
Intracellular cGMP synthesis in fractionated lymphocytes
Lymphoid cell transcription and translation of DNA
Inhibition of an early stage of B-cell differentiation
Induction of a late stage of B-cell differentiation
Induction of complement receptor on human granulocytes
In vivo activities:
Delay of impairment of neuromuscular transmission in the mouse and rat
Induction of prothymocyte cells of nude mice with appearance of cells with TL^+ Thy 1^+ phenotype in the spleen and TL^- Thy 1^+ in lymph nodes
Delay of the onset and reduced severity of autoimmune hemolytic anemia in NZB mice
Enhancement of lower than normal MLR of lymph node cells of NZB mice with established autoimmune disease
Enhancement of the reduced T-cell-dependent antibody responses of 2-year-old mice

conducted research on the biological activities of the pentapeptide representing residues 32–36 of TP in a model designed to examine whether the thymus gland or its products can ameliorate or prevent the immune deficiencies that accompany aging. Spleen cells from aged mice exposed either in vitro or in vivo in the synthetic pentapeptide fragment of TP showed partial restoration of the number of antibody-forming plaque cells and high affinity PFC to the antigen dinitrophenylated bovine gamma globulin. Their data indicate that this pentapeptide fragment of TP may enhance the capacity of aged mice to retain in part their immune function.

The activity of this pentapeptide is particularly noteworthy in view of the previous observation from the same group of investigators (*144*) that synthetic tridecapeptide, corresponding to residues 29–41 of bovine TP, exhibited only 3% of the activity of the parent polypeptide in stimulating the conversion of prothymocytes to thymocytes. Although the pentapeptide does represent a segment of the tridecapeptide, these investigators concluded in an earlier publication that "the tertiary structure of thymopoietin may be required for optimal configuration of the active site to produce full biological activity." Perhaps the parent molecule and the small fragments may differ in activity from one another in specific assays.

Thymic Factor X

Chemistry

The role of immunological mechanisms in the pathogenesis of leukemia and their effects on the course of the disease have been a subject of study by Aleksandrowicz since 1948 (*20*). In view of later observations indicating the involvement of the thymus in hematopoiesis, the institute at which he worked initiated studies during 1972 to 1974 with the goal of stimulating host immunological mechanisms. In initial efforts, thymus fragments taken from patients with myasthenia gravis were transplanted into selected patients with leukemia and Hodgkin's disease. The appearance of immunological enhancement a short time after the thymus transplant led the group in Krakow to begin work in 1973 on the clinical usefulness of calf thymus extract.

In the early studies, an aqueous extract of calf thymus tissue, TFX, was used. Evidence that the administration of this factor resulted in enhancement of humoral immunity and CMI led to attempts by Szmigiel and Rzepecki and their colleagues (*145, 146*) to purify the crude aqueous preparations of TFX.

Skotnicki (*21*) published a procedure that resulted in a highly purified TFX. The aqueous extract of thymus glands from 5- to 7-week-old calves was subjected to ammonium sulfate fractionation, ion-exchange chromatography, and precipitation with ethyl alcohol in an acidic medium. The insoluble fraction was a protein that was homogeneous on PAGE. The isolated molecule was free of lipid, carbohydrate, and nucleotide. Electrophoresis with 0.1% SDS at pH 6.8 indicated a mol wt of approximately 4,200. The amino acid composition of TFX is presented in table 4-17.

Comparisons of the amino acid composition of TFX, thymosin α_1, TP,THF, and a lymphocytopoietic factor (r) show no evident similarity among these various preparations. In the absence of sequence analysis of TFX and THF, the data available for TFX cannot contribute to clarification of the question regarding possible chemical relationships among the putative thymic hormones.

Biology

The biological activities of the calf thymic product TFX [both the earlier preparations and the reported homogeneous product (*21*)] have been studied in in vitro and in vivo assays. Table 4-18 lists some of the more significant activities described for it.

TABLE 4-17.—*Amino acid composition of TFX*[a]

Amino acid	nmol/100 nmol protein
Aspartic acid	7.0
Threonine	4.2
Serine	4.1
Glutamic acid	11.4
Proline	11.9
Glycine	25.7
Alanine	8.0
Valine	3.2
Methionine	1.0
Isoleucine	3.9
Leucine	5.3
Tyrosine	1.2
Phenylalanine	1.8
Histidine	1.2
Lysine	5.2
Arginine	4.9

[a] *See* (*21*).

TABLE 4-18.—*Some biological activities of TFX*[a]

In vitro activities:
Restoration of the azathioprine sensitivity of spleen rosette-forming cells from adult thymectomized mice
Enhanced response to mitogens of lymph node cells from mice treated in vivo with TFX
Increased rosette formation by human cord blood lymphocytes
Increased levels of intracellular cAMP and protein kinase of blood lymphocytes of patients with CLL
In vivo activities:
Increased survival of mice exposed to gamma radiation with an increase in the LD_{50} dose
Increase in number of blood T-lymphocytes and return of delayed hypersensitivity (human)

[a] *See (21, 29).*

Thymostimulin

This thymus extract was prepared initially by Bergesi and Falchetti (*147*) and has also been termed "TP-1" (*148*). It is prepared as follows: Calf thymuses are minced and extracted with ammonium acetate. The extract is heated to 70° C, filtered, and precipitated with ammonium sulfate. Then the precipitate is dissolved in water and subjected to ultrafiltration on an Amicon PM-10 membrane. The filtrate is desalted on Sephadex G-25 and gel-filtrated on Sephadex G-50. On PAGE, the fractions that showed 2 characteristic bands with RF 0.22 and 0.42 were combined and called TP-1 (TS).

Porcine Thymic Hormone

Since 1977, investigators at the Laboratory of Molecular Biology in Nanking University, Nanking, China, prepared thymosin fraction 5-like material from porcine thymus (*31*) and used a modified E-rosette assay to follow the purification.

The crude extract of pig thymus is partially denatured by heat, followed by ammonium sulfate fractionation, and finally purified by DEAE-cellulose chromatography. This partially purified thymic hormone preparation is free from carbohydrates and nucleotides and contains at least 8–9 protein components that have mol wt from 9,000 to 68,000 and isoelectric points that range from 5.0 to 7.5.

The porcine thymic hormone fraction 5 thus prepared exhibits extremely high biological activity as demonstrated by a microrosette assay. Data reported showed that this thymic preparation was active at 0.1 μg/100,000–200,000 lymphocytes in inducing the maturation of T-cell populations (*31*).

Two components designated porcine thymic hormones T_1 and E_1 (*149*) have been purified from the crude preparation. They are acidic proteins with mol wt of 13,000 and 68,000, respectively. The isoelectric point of T_1 was determined as 4.6 and E_1 as 5.2.

The porcine thymic hormone may be useful in the treatment of primary immunodeficiency; autoimmune disorders; certain neoplastic diseases; and various bacterial, viral and fungal infections (*31*). Treatment of some patients with the porcine thymic hormone at 4 mg/day, three times/week im for 1 to 3 months resulted in increases in T-cell percentages (*31*).

Calf Thymic Preparation

Liu and co-workers (*32*) at the Chinese Academy of Medical Sciences, Beijing (Peking), China, prepared thymosin fraction 5-like material from calf thymus. A modified rosette assay that required human umbilical cord blood lymphocytes was used for the purification steps. The purification consists of homogenization, an 80° C heat step, an ammonium sulfate precipitation step, and gel filtration on Sephadex G-50.

Reports of the biological activity with this preparation in the literature to date are limited, but a recent one that described the clinical effects of the calf preparations in patients with opportunistic infections (*33*) is interesting. Most of the patients were reported to have serious opportunistic infections, including encephalomyelitis, hepatitis, herpes zoster, and fungal infections associated with immunosuppressed states secondary to cancer. Lately, an additional 20 patients with assorted opportunistic infections were treated, of which 17 have had significant clinical improvement (Liu S-L: Personal communication).

Other Thymic Factors

Homeostatic Thymic Hormone

Chemistry.—Some of the earliest studies of the activity of a cell-free thymic extract were those of Comsa (*150–159*), who described the presence in thymic extracts of a fraction that showed antagonistic effects initially against thyroxine in normal and hypophysectomized rats and subsequently against a number of other hypophysical hormones, including adrenocorticotropic and thyrotropic hormones and the gonadotropins. In contrast to these antagonistic effects, the homeostatic thymic hormone had a synergistic effect with growth hormone. Pertinent to our present considerations of the chemistry of thymic fractions was the report by Comsa (*156*) that this hormone exhibited chemotactic activity on lymphocytes and restored antibody production in thymectomized guinea pigs and delayed hypersensitivity to thymectomized rats.

A description of the preparation of the homeostatic hormone from calf thymus that has been published (*158*) can be summarized as follows: Ground calf thymic tissue is extracted with 1 *M* hydrogen sulfate, and the extract is neutralized with ammonium hydroxide and half saturated with ammonium sulfate. The supernatant from the precipitated proteins is brought to full saturation with the latter chemical. The precipitate is dissolved in 0.03 *M* HCl, then brought to pH 7.0 with ethanolic ammonia. The ethyl alcohol concentration is brought to 20%, the solution adjusted to pH 6.2 with HCl, and the alcohol concentration increased to 66%. The precipitate is subjected to successive chromatography on Sephadex G-25 and hydroxyapatite, followed by desalting on Sephadex and lyophilization to yield an apparently homogeneous product with a mol wt between 1,800 and 2,500. Apparently, the product is a glycopeptide because, on hydrolysis, sugars and amino sugars in addition to amino acids were present in the

hydrolysate. The final product mimicked the biological activities of the earlier, less purified preparations. The composition and sequence of this homeostatic hormone are awaited with interest particularly because this molecule appears to be the only described thymic hormone-like product in which carbohydrate is present.

Biology.—The glycopeptide isolated by Comsa and his co-workers (*158*) was studied extensively with regard to its biological activities (table 4-19), especially those interrelated with the actions of other endocrine glands. The activity of this product in immunological phenomena is well documented.

Lymphocytopoietic Factors

Chemistry.—Two protein components, isolated from bovine thymus by Luckey and his colleagues (*160*), have known activity in promoting immune competence when injected into neonatal mice. In these investigations, they used 3 assays to follow the biological activity. One involved the lymphocyte-to-PMN cell ratio in the blood of newborn mice, with assessment of alterations in this ratio after administration of a fraction from bovine thymus. Although this assay is not a critical measure of lymphocytopoiesis, in that an absolute lymphocytosis would be of greater significance, it was useful in the initial work. Of the fractions studied, 2 homogeneous proteins were obtained and designated as lymphocytopoietic factors r and h. These two proteins had mol wt of 80,000 and 15,000, respectively; the r was described as heat stable and the h factor as heat labile. The products are free of carbohydrates. Each protein appeared to have lymphocytopoietic activity in the neonatal mouse assay. Table 4-20 presents the amino acid composition of the 80,000-dalton protein.

Further physical and chemical characterization of these proteins and an indication of their sequence will at some later date aid in further clarification of their relationship to other postulated thymic hormones.

Biology.—To the present time, the published biological data of the 2 lymphocytopoietic factors described by Luckey and his associates are limited. Both preparations assayed in newborn mice enhanced antibody production to SRBC and induced a lymphocytosis. The latter reaction was based on an increase in the ratio of blood lymphocytes to PMN cells, rather than the more critical parameter of absolute numbers of blood lymphocytes as a percentage of total leukocytes.

TABLE 4-19.—*Some biological properties of homeostatic thymus hormone*[a]

Suppression of deleterious consequences of thymectomy in young (12- to 16-day-old) guinea pigs, e.g., restoration of weight gain and ability to produce antibodies
Restoration of delayed hypersensitivity to thymectomized rats
Positive chemotactic influence on lymphocytes of male rats
Antagonistic with adrenocorticotropic, thyrotropic, and gonadotropic hormone action; synergistic with growth hormone
Diminution of the radiation syndrome in guinea pigs

[a] *See* (*159*).

TABLE 4-20.—*Amino acid composition of the 80,000-dalton lymphocyte-stimulating hormone factor r*[a]

Amino acid	Percent	Amino acid	Percent
Alanine	6.14	Lysine	5.94
Arginine	5.72	Methionine	1.22
Aspartic acid	9.70	Phenylalanine	5.97
Cysteine–cystine	1.38	Proline	6.06
Glutamic acid	16.65	Serine	5.68
Glycine	2.55	Threonine	5.37
Histidine	4.46	Tryptophan	Present
Isoleucine	2.72	Tyrosine	4.21
Leucine	10.34	Valine	5.95

[a] *See* (*160*).

Hypocalcemic and Lymphocytopoietic Substances

Chemistry.—In 1944, Ogata, Ito, and Mizutani reported that a fraction from calf thymus gland lowered serum calcium in rabbits. Since that initial observation, Mizutani and colleagues (*161*) continued their studies of the purification of the hypocalcemic substance from the thymus. With the development of knowledge of the important role of the thymus in immune reactions, these investigators turned their attention to the purification and characterization of their fractions, using hypocalcemic and lymphocyte-stimulating assays.

In the course of their research, Mizutani and his associates isolated from bovine thymus extracts 2 hypocalcemic factors which they designated as TP_1 and TP_2. A saline extract of an acetone-dried powder of thymus tissue was fractionated with ammonium sulfate. These 2 factors were obtained by chromatography on DEAE cellulose of the fractions precipitated at 15 or 20% concentrations of ammonium sulfate. Fraction TP_1 was subjected to successive chromatography on Sepharose 6B, followed by preparative PAGE. The isolated product, TP_1, yielded a single band on analytical gel electrophoresis.

Fraction TP_2 was also purified by chromatography on Sepharose 6B and gel electrophoresis. The final TP_2 product was obtained by removal of the acrylamide by dialysis, lyophilization of the aqueous extract, and removal of the final polyacrylamide gel particles by means of gel chromatography on Sepharose 6B (*161*).

Biology.—The biological activities of the 2 factors prepared from thymic tissue by Mizutani and his associates (*161*) include 1) hypocalcemic activity in normal rabbits, 2) enhanced production of antibody to SRBC in neonatal mice, and 3) a relative lymphocytosis according to the method of Luckey and his co-workers.

Thymic Epithelial Supernatant

Chemistry.—Several investigators reported the presence of thymic hormone-like activity in the cell-free supernatant of cultures of thymic epithelial cells. The most extensive biological studies of such preparations are those of Kruisbeck and her colleagues (*162, 163*).

Thymic tissue from 5-week-old female rats is dissected into small fragments, and the tissue fragments are washed several times with RPMI-1640 buffered with 25 m*M* HEPES. This procedure removes most of the lymphocytes.

Centrifugation between washings yields tissue fragments which are then cultured in a medium consisting of HEPES-buffered RPMI supplemented with 2 m*M* glutamine, 100 IU penicillin/ml, 0.1 mg streptomycin/ml, and 10% heat inactivated rat serum. After 26 hours at 37° C, extra medium is added to give a final culture volume of 10 ml/flask; each flask contained fragments from a single thymus. The cultures are lymphocyte free after the first or second change of media.

Media collected from the cultures is centrifuged at 700×*g* for 10 minutes at 4° C, the supernatants are filtered through Millipore filters (0.45 μ), and stored at −20° C. This crude preparation, tested in various assays that exhibited the chemical nature of the factor or factors responsible for the biological effects of thymic epithelial supernatant, awaits characterization.

Biology.—This supernatant described by Kruisbeck and her associates (*162*), when added to cultures of rat thymocytes, increased dThd incorporation into these cells in the presence of PHA or Con A. The increased mitogen responsiveness was at least partially due to an increase in the number of responsive cells. In addition, the data suggested that the target cell for thymic epithelia supernatant is a cortisone-sensitive thymocyte that had little effect on mitogen-stimulated spleen and lymph node cultures. Later studies of the activities of this supernatant in vitro (*163*) demonstrated a stimulatory effect on the fraction on the MLR and on antibody production to SRBC by spleen cells from nude mice.

Polypeptide Thymic Extracts

Since 1949, investigators at the Institute of Endocrinology of the Academy of Medical Sciences in Bucharest have been examining the chemical and biological properties of an aqueous extract prepared from a lipid-free powder of fresh calf thymus. This powder was subjected to a brief alkaline hydrolysis, neutralized to pH 7.0, and lyophilized. This fraction was a thymic polypeptide preparation (*164*).

On electrophoretic analysis with the then available Tiselius apparatus, the pattern obtained suggested a single product. However, chemical analysis, though indicating the absence of proteins, revealed the presence of amino acids and low molecular weight polypeptides. Paper chromatography of the extract gave 13 spots, which, on comparison with standards, confirmed the presence of amino acids in addition to the polypeptides. These were cysteine, arginine, histidine, lysine, aspartic acid, glycine, glutamic acid, threonine, alanine, proline, tyrosine, methionine, valine, leucine, and isoleucine. This relatively crude extract could be heated to boiling with no apparent loss of biological activity.

A similar polypeptide extract was derived from horse embryo thymus collected during the seventh month of gestation. The final extract, assessed on paper chromatography, revealed the presence of the same amino acids (qualitatively) as those derived from calf thymus but in reduced quantities.

The biological properties of the thymic polypeptide preparation described by Potop and Milcu (*164*) have been studied in some detail. Some of the reported results are listed in table 4-21. In most of these results, the experimental model involved use of thymectomized animals and adequate control studies with, generally, muscle extracts prepared similarly to the extract used for the preparation of TP.

Nonpolar Extract: Thymosterin

Chemistry.—Studies initiated by Jankovic (*165*) and his colleagues in 1965 first drew attention to the possibility of lipid fractions of the thymus having immunological potential in neonatally thymectomized rats.

Thymus glands obtained from 18- to 28-day-old rats were homogenized in saline followed by lyophilization of the homogenate. The dried residue was extracted for 12 hours with hot chloroform–methanol. As a control, CNS tissue from rats was treated similarly. After removal of the solvent, the residues were emulsified in saline prior to injection. The demonstration that the thymic lipid fraction was active in promoting growth and restoring immune potential in neonatally thymectomized rats led to the conclusion that the reported humoral activity of the thymus appeared to be related to the lipid, lipoprotein, or lipid-soluble components of the gland.

Earlier experiments of Potop and Milcu (*164*) in 1961 described a total lipid extract of the thymus, which, when administered to rats bearing an MCA-induced tumor, had significant antitumor activity. Subsequently, purification of this lipid extract was achieved beginning with lyophilized frozen minced thymus, which, with ethyl ether, yielded a dry residue on evaporation of the extract in a vacuum. This fraction was termed the "total lipid extract."

By use of selective organic solvent fractionation, 4 lipid-containing fractions were isolated from calf thymus. One of these, named "fraction B," was then separated into 3 fractions by standard procedures for the separation of blood steroids. A fraction designated as IIB had significant antitumor activity and, on analysis by gas chromatography,

TABLE 4-21.—*Some biological properties of thymic polypeptides extract*[a]

Stimulation of Ca^{2+} and PO_4^{3-} deposition in bones of normal rabbits and in rabbits previously subjected to X-ray irradiation of the thymus
Elevation of serum calcium concentration and decrease in serum inorganic phosphate
Restoration to normal of the depressed liver catalase activity and blood hemoglobin levels of thymectomized rats
Increase in synthesis and metabolism of nucleic acids and proteins in the livers of thymectomized rats
Alterations in carbohydrate metabolism, i.e., stimulation in liver of glycogen synthesis, glucose-6-phosphatase and fructose 1,6-bis phosphatase activities, and ATP synthesis
Antiproliferative action on KB tumor cells in vitro
Inhibition of tumor growth induced in rats by MCA or DMBA
Decrease in hemagglutination titers in lung cell suspensions of mice inoculated with influenza virus
Stimulation of antibody synthesis in immunized, X-ray irradiated rabbits and in normal rats inoculated with A_2 influenza vaccine
Increased survival in mice inoculated with *Salmonella enteritidis*

[a] *See* (*164*).

contained a high proportion of saturated fatty acids, particularly palmitic and stearic acids, and also significant amounts of oleic acid. This fraction was rich in cholesterol (approximately 58 mg/g fraction).

In contrast, fraction IIIB had only trace quantities of cholesterol but contained lipids, which, on chromatography, exhibited the characteristics of steroids. Countercurrent distribution and chromatographic analysis of this fraction ruled out the presence of androgens or estrogens and indicated only a trace of corticoids.

Fraction IIB was further purified by both silica gel and thin-layer chromatography to yield subfractions designated as IIB_2, IIB_3, IIB_4. In vitro assay of these fractions with KB tumor cell cultures suggested antiproliferative activity in each fraction; however, fraction IIB_3 was distinctly the most active.

Infrared spectroscopy of fraction IIB_3 revealed the presence of a steroid nucleus, as well as 12-acetoxy-11 ketones. Further purification of fraction IIB_3 led to the isolation of a compound designated as factor S, which had significant in vitro antiproliferative activity. Examination of the structure of the pure factor S indicated a C_{28} steroid with methyl groups at C-21 and C-28. The infrared spectrum also showed the presence of a hydroxyl group bound to the steroid nucleus and either a ketone or an acetate carbonyl in the molecule with the suggestion that the ester was a methyl ester. In view of this structure, the molecule was named "thymosterin."

Biology.—Potop and her colleagues presented data for the biological activity of purified thymosterin (factor S). This product had antiproliferative action on KB tumor cells in culture (*164*). The inhibition of dThd incorporation was accompanied by decreases in the following cellular enzymes: lactate dehydrogenase, malate dehydrogenase, glucose-6-phosphate dehydrogenase, and alkaline phosphatase.

These investigators also reported that their nonpolar fraction from thymic tissue effectively repaired the specifically altered metabolism of normal or thymectomized tumor-bearing or X-ray-irradiated animals and enhanced immunological responsivity, hemoglobin, and lymphocyte numbers in neonatal animals (*164*).

THYMIC HORMONE-LIKE FACTORS ISOLATED FROM BLOOD

Facteur Thymique Serique From Pig Serum

Chemistry

In the eourse of early interest in the assessment of the immunological status and the relative likelihood of kidney rejection in patients with renal transplants, Bach (*43-45*) developed what has now become a rapid, useful method for the detection of the level in human plasma of thymic hormone-like activity. By the application of this sensitive assay, Bach and his associates established the correlation of this serum activity in mice with both immunological competence and the presence of a functioning thymus gland. Furthermore, he and his colleagues (*43-45, 60, 68*) isolated from pig serum the factor that is active in their assay and also exhibits a number of other biological features.

Porcine blood was chosen as the starting material. Ultrafiltration procedures that required the use of Amicon membranes produced a significant concentration of the active product by membranes that retained molecules with weights greater than 10,000. This Diaflow ultrafiltration system was utilized initially for extensive concentration (approximately 100-fold) of the active factor.

To process large volumes of pig serum, Bach et al. used a special apparatus that essentially involved ultrafiltration of defibrinated pig blood with subsequent concentration of the serum ultrafiltrate in an Amicon diafiltration chamber. This procedure resulted in a concentration of activity of approximately 25,000-fold. The pooled concentrated samples were taken up in phosphate buffer and then subjected to Sephadex G-25 chromatography. The active fractions from Sephadex chromatography were eluted with phosphate buffer (0.2 *M*, pH 7.3), with a further increase in activity of approximately tenfold. Desalting by use of Amicon membranes and application to a CMC column equilibrated with phosphate buffer (0.01 *M*, pH 6.3) followed by elution of the active fraction with an NaCl gradient yielded a further increase of activity (~twofold). The active fraction was again desalted on Amicon membranes and chromatographed on Sephadex G-25 in 1 *M* acetic acid. The fraction containing FTS activity, obtained by this step, was subjected to gel filtration on Sephadex G-10 in aqueous medium.

Molecular weight as determined by the elution position of FTS on a column of Sephadex G-25 in 1 *M* acetic acid, compared with a number of peptides of known molecular weight, suggested a value of 900 for FTS. Electrophoretic mobility of FTS at varying pH values indicated a neutral isoelectric point for the molecule.

From the initial 15 liters of normal pig serum containing 1,200 g of total protein, the yield of active peptide was 3 μg. Assessed by the in vitro rosette assay, the biological activity had been increased approximately 100,000-fold.

Amino acid composition followed by sequence analysis established the following structure for FTS (*60*):

Glutamic acid–alanine–lysine–serine–glutamine–glycine–glycine–serine–asparagine.

The synthetic and isolated natural FTS exhibit comparable biological activities (*64*) in those in vitro and in vivo assays in which they were compared.

Bach and his colleagues (*166*), using an RIA for the evaluation of serum FTS levels, reported the presence of FTS in pig thymic tissue. The latter was at least ten times higher than pig serum in the concentration of FTS. Bach also detected FTS in calf thymosin fraction 5 as assessed by RIA and biological activity (personal communication). This suggests that FTS or a precursor product is derived from the thymus. However, the antiserum Bach used was raised in the rabbit against the porcine FTS, coupled as a hapten to BSA, and applied to a fraction from calf thymus. The significance of this RIA applied to the heterogeneous fraction 5 requires further examination.

Biology

The principal biological effects of FTS are summarized in table 4-22. It is evident from the data that the partially purified and pure circulating hormones isolated by Bach and co-workers produce responses both in vitro and in vivo that are characteristic of thymic hormones. The increases in anti-DNA antibody production and in glomerulonephritis in B/W mice are worthy of attention. This was seen after administration of larger doses of FTS, which suggested a decrease in suppressor cell numbers or function.

Protein Fraction From Human Plasma (Prealbumin)

Chemistry

Thymic hormone-like activity has been detected in the blood of several species. As indicated above, FTS, a nonapeptide with such activity, has been isolated from porcine blood and its structure was established. The demonstration of thymic hormone-like activity in human blood (*43, 166*) led Burton et al. (*167*) to isolate the active component.

Gel filtration of fresh human serum on Sephadex G-150 revealed that approximately 95% of the total azathioprine-sensitive, spleen cell rosette assay activity was present in a fraction that coincided with the albumin and prealbumin peaks. Only traces of activity (less than 5%) were recovered in the lower molecular weight fractions. The peak containing the major portion of the total serum activity corresponded to a fraction with a mol wt of approximately 52,000. With analysis by electrophoresis, this fraction appeared to be composed largely of albumin and small amounts of prealbumin.

TABLE 4-22.—*Some biological properties of FTS*[a]

Stimulation of expression in vitro of selected T-cell specific antigens on normal bone marrow and on spleen rosette-forming cells following injection of FTS into either adult thymectomized or athymic mice
Restoration of responsiveness of spleen cells of adult thymectomized rats and NZB mice to mitogens (PHA and Con A) after in vivo administration of FTS
Restoration of the capacity of adult thymectomized mice to generate cytotoxic lymphoid cells following treatment in vivo with FTS
Restoration of capacity of thymectomized, irradiated mice to restrain growth of a Moloney virus-induced sarcoma following treatment with FTS
Enhancement of the generation of effector cytotoxic T-cells both in vitro and in vivo
Inhibition of antibody production in NZB mice challenged with a thymic-independent antigen, e.g., polyvinyl pyrrolidone
Inhibition of contact sensitivity in normal mice
Delay of allogeneic skin graft rejection in adult mice
Prevention of the appearance of autoimmune hemolytic anemia and Sjögren syndrome in NZB mice
Increase in anti-DNA antibody production and in incidence of glomerulonephritis in B/W mice
Induction of the transformation of cortisone-sensitive thymocytes into cortisone-resistant thymocytes
In vitro inhibition of dexamethasone uptake by thymocytes

[a] *See (43, 44, 166).*

The purification procedure developed for isolation of the thymic hormone-like activity from pooled human plasma is diagrammed in figure 4-9. Inasmuch as human serum albumin and prealbumin are present in fraction IV-5 in modifications of the procedure described initially by Cohn and his associates, this fraction was used as the starting material. Each step was performed at 5° C unless otherwise specified. Generally, 40-g aliquots of lyophilized Cohn fraction IV-1 of pooled human plasma were stirred for 3 hours at room temperature with 1 liter of 50 m*M* Tris buffer (pH 8.0) containing 0.02% sodium azide. The suspension was centrifuged at 13,000×*g* for 30 minutes. Solid ammonium sulfate was then added to the supernatant to 40% saturation. The precipitate was allowed to settle and then was centrifuged at 13,000×*g*. The ammonium sulfate concentration of the supernatant was then brought to 60% saturation, and the precipitate was collected, dissolved in cold distilled water, and lyophilized (fraction 1). Two successive chromatography steps on the Sephadex G-150 column equilibrated with 50 m*M* Tris/100 m*M* NaCl/0.02% sodium azide (pH 8.0) provided a fraction weighing about 40,000 to 68,000 daltons. This material was collected, desalted by diafiltration through an Amicon UM-10 membrane, and lyophilized. The active fraction was subjected to preparative gel electrophoresis at pH 8.9 in a Canalco apparatus with cooled gel columns. The active fraction was desalted by diafiltration, followed by gel filtration on a small Sephadex G-150 column equilibrated with 20 m*M* ammonium bicarbonate (pH 7.8). The peak containing the biological activity (fraction 4) was collected and lyophilized. Yields and relative activities of each fraction are summarized in table 4-23. It may be noted that the high degree of enhancement of activity is probably partly due to the partial removal by the 40% ammonium sulfate saturation step and the 2 subsequent Sephadex G-150 chromatography steps to a protein fraction that is inhibitory in the rosette assay.

Fraction 4, the final product, behaves as a homogeneous protein on analytical PAGE at pH 3.9. The mobility of this fraction and its amino acid composition were identical with preparations of authentic prealbumin (*167*). Thus the homogeneous protein obtained from pooled human plasma exhibited a mol wt of 56,700±300 when assessed by sedimentation equilibrium in the ultracentrifuge. Electrophoresis of fraction 4 in SDS gels revealed the presence of a major band of 52,000 daltons, which indicated that the molecule consisted of 4 subunits of identical size. Authentic samples of prealbumin behaved in a similar manner.

Immunodiffusion experiments against prealbumin antiserum with fractions 3 and 4 and with a commercially obtained prealbumin resulted in the formation of single confluent precipitant arcs. Amino terminal group analysis showed a glycine as the only amino terminal residue in fraction 4 and in authentic prealbumin.

Assessment of the activity of fractions 3 and 4 by the in vivo reconstitution of the azathioprine sensitivity of the spontaneous rosette-forming spleen cells of adult thymectomized mice indicates that 2 g of fraction 4 or 100 g of fraction 3 administered ip for 3 days recon-

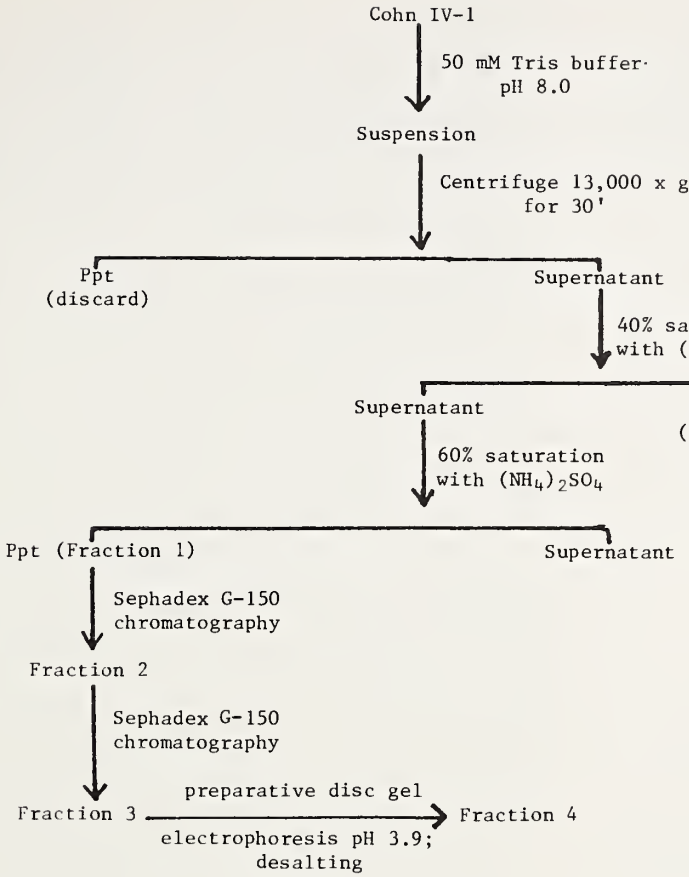

FIGURE 4-9.—Isolation of prealbumin fraction from human plasma.

stituted the azathioprine sensitivity of the spontaneous rosette-forming spleen cells to normal or above-normal values. Retinol, which binds to prealbumin in the presence of retinol-binding protein, was inactive at the doses tested (*167*). Thyroxine, as well as retinol or retinyl acetate, previously indicated to be transported by prealbumin, were not active in the azathioprine assay (White A: Personal communication).

We wish to emphasize that, presently, no chemical relationship of prealbumin to either the Bach FTS nonapeptide or to the thymus-dependent human SF described by Astaldi and his colleagues is evident. The in vitro activity of the synthetic FTS reported by Burton et al. (*167*) is distinctly less than that described by Bach and his associates (*166*). However, until each of the synthetic products prepared by these 2 groups of researchers is compared by each of them (and preferably at the same time) with isolated FTS, it is premature for anyone to draw conclusions regarding the possible relationships of the 2 blood products. Of present significance for thymic biology are the activities in vivo of the isolated products.

TABLE 4-23.—*Purification of prealbumin from Cohn fraction IV-1 of pooled human plasma*[a]

Fraction	Weight, g[b]	Activity, ng protein	Enhancement of activity[c]
Cohn IV-1	40	12,000	1
1	17	1,000	12
2	4	300	40
3	2	30	400
4	0.02	0.03	40,000

[a] *See* (*167*).

[b] Minimum weight of material necessary to reduce by 50% the number of SRBC rosettes formed in vitro by spleen cells from adult thymectomized mice in the presence of a constant amount of azathioprine.

[c] Activity enhancement was relative to that of initial Cohn IV-1 fraction.

Biology

The partially purified fraction from human plasma described by Burton et al. (*167*) has been subjected to several assays in normal and in neonatally thymectomized mice, the results of which are summarized in table 4-24.

Thymus-dependent Human Serum Factor

Chemistry

Astaldi and his colleagues (*168–170*) demonstrated the presence in human serum of a thymus-dependent factor which they designated as "SF." This factor was characterized by its ability to increase the intracellular cAMP level of human and mouse thymocytes when the cells were incubated with aliquots of human serum. The target cell for this factor was restricted to thymocytes; no effect was observed with normal human PBL or other lymphoid and nonlymphoid cells.

TABLE 4-24.—*Biological activities of protein fraction from human plasma*[a]

Increase in numbers of azathioprine-sensitive E-rosette forming spleen cells from adult, thymectomized mice following either in vitro incubation of cells with the fraction of injection of the latter followed by in vitro assay
Enhanced survival of neonatally thymectomized mice
Enhanced ability of neonatally thymectomized mice to reject an allogeneic skin graft
Increase in numbers of sheep erythrocyte PFC (IgM antibody) in vitro by spleen cells from neonatally thymectomized mice treated in vivo with the blood fraction
Enhanced MLR of mouse spleen cells incubated with the blood fraction
Decrease in rate of growth to a slow-growing, mammary adenocarcinoma and enhanced survival of DBA/2 female mice treated in vivo with blood fraction prior to tumor transplantation

[a] *See (167).*

The stimulation of synthesis of cAMP by this factor was no longer observed when normal mouse thymocytes were depleted by hydrocortisone-sensitive cells (*169*). Furthermore, incubation of thymocytes with it increased the population of hydrocortisone-resistant cells. We interpreted the data as indicating that SF acts on hydrocortisone-sensitive cells, increasing their intracellular cAMP and inducing their transformation to hydrocortisone-resistant cells.

The thymic origin of SF is inferred from the following observations: Although SF was present in the sera of normal donors, it declined progressively after donors reached the age of 30 years. It was not detectable in thymectomized human donors or in patients with thymus-dependent immunodeficiency diseases. Some of the SF activity may by due to a 500-dalton peptide (Astaldi A: Personal communication) and is distinct from that obtained from pig serum by Bach and his co-workers. Most recently, Astaldi reported that the SF activity associated with the stimulation of cAMP was due to adenosine (*171*).

Biology

The SF of Astaldi et al. (*168–170*) acts selectively on hydrocortisone-sensitive thymocytes, stimulates protein synthesis when added in vitro to the cells, and induces human lymphocytes to express an F_c receptor for IgM but not IgG. In normal human thymocytes, the TdT was decreased in activity following incubation of the cells with the factor (*169*). The authors concluded that SF acts on thymocytes to induce select cell numbers to acquire some properties of mature T-cells. This conclusion is in harmony with the observation of Hu et al. (*117*) that TdT is turned off by thymosin α_1 during the transformation of the prothymocyte into a mature T-cell. The data would suggest that Astaldi's factor acts on T-cells during the later stages of maturation.

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SECTION V

Augmenting and Immunomodulating Agents:

Illustrative Examples

BCG AND BCG FRACTIONS

Introduction

An attenuated strain of *Mycobacterium bovis,* BCG was developed as a vaccine against human tuberculosis by Calmette and Guérin at the Pasteur Institute in 1908. Particularly within the past 20 years, BCG has received considerable attention in completely different roles as an immunological modulator and an antitumor agent. Investigations with this agent in the immunotherapy of cancer have met with some success in different animal systems and with limited success in several trials in man. The majority of this work has been done empirically with the regression of the tumor or survival of the animal as the end point and with relatively less attention to measurement of antitumor immunity. Because the effects of BCG on the immune response are multifaceted, a clearer understanding of what is known of its mechanisms of action may lead to a more rational basis for its use, not only in cancer immunotherapy but also in other immunological applications.

In this review, we will first describe some of the effects of BCG on broad phenomena, such as the rejection of tumors and the synthesis of antibody and CMI, and then try to indicate what is known of the specific cellular mechanisms by which it may act.

Antitumor Effects

It is an interesting irony that, although the antitumor effects of BCG have rekindled scientific interest in the whole field of immunological modulation, it is precisely those effects that are the least well understood immunologically. The complexity of the immune response to tumors and the wide variation in modes of administration of BCG account for much of the difficulty. Although our subsequent discussion of the effects of BCG on defined populations of lymphoid cells may shed some light on this issue, a general categorization of the antitumor applications may be useful because of their practical importance.

Several broad distinctions might be made. It is important to distinguish between the use of BCG directly into a site of tumor growth (intralesional or intratumoral BCG) and BCG given by vaccination into uninvolved skin or by injection (systemic BCG). In addition, the administration of BCG as a prophylaxis during the period of induction of a tumor or before an inoculum of cells has become a palpable tumor is distinct from therapy of an established tumor. Finally, the use of BCG alone as a nonspecific stimulator of the RES or of immunity should be distinguished from its use as an adjuvant, i.e., in conjunction with antigen (tumor cells). The importance of recognizing such categories as these is considerable when one is attempting to decide whether on balance BCG is a significant antitumor agent because it is not a cytotoxic chemical but simply a modifier of the host's intrinsic antitumor response, the effects of which are highly dependent upon scheduling.

Nearly 20 years ago, Old et al. (*1*), Biozzi and coworkers (*2*), and Halpern and associates (*3*) independently showed that BCG increased the resistance of rats and mice to several transplantable tumors, especially lymphomas. Given before or concomitantly with the tumor, BCG was an effective immunoprophylactic agent. Mathé et al. (*4*) later began treatment of mouse leukemia L1210 with BCG immediately after inoculation of the cells and found that 20% of the mice could be cured if 10^4 tumor cells or fewer were used. Leukemia–lymphomas in mice are the most responsive of tumors to prophylaxis with BCG; Mathé and associates (*5*) prolonged survival by systemic pretreatment with BCG before L1210 and also found the established lymphomas frequently responsive to BCG and many other adjuvants (*6*). The growth of mouse lymphomas has either been inhibited by BCG or unaffected but never enhanced, whereas solid tumors have been enhanced occasionally.

When given systemically, BCG inhibited the induction of tumors by MCA (*7, 8*) if mice were treated 2 weeks before or after the application of the carcinogen. When the tumors became palpable, treatment was usually ineffective. The growth of MCA-induced tumors was not prevented completely but was at least considerably delayed, even in experiments in which the final incidence of tumors was the same as in controls (*7*). A transient suppression of carcinogen- and virus-induced tumors was also reported earlier by Old et al. (*9*). The incidence of spontaneous AKR leukemia was prevented by repeated injection of BCG (*10*), and MER prevented radiation virus-induced leukemia (*11*). All these data on systemic prophylaxis suggest that many of the effects of BCG on tumors cannot be explained by local granuloma formation and nonspecific destruction of "innocent" bystander tumor cells, factors which may partly account for its intralesional effects. In fact, Old et al. (*9*) attributed the prophylactic effects to systemic stimulation of the RES, which in broad outline may be correct, with cellular details that we will consider shortly.

Much of the work on immunotherapy of established tumors by BCG has dealt with direct intralesional injection, especially with the transplantable line 10 hepatocarcinoma in the guinea pig (*12–14*). This tumor, when injected intradermally into the animal, grows progressively, spreads first to regional lymph nodes and is weakly antigenic for the syngeneic host, thus mimicking many of the attributes of human tumors. The growth of established tumor grafts would be suppressed by intralesional injection of living BCG organisms (*12–16*). Bekierkunst (*17*) achieved similar results with killed BCG coated with cord factor (trehalose-6,6′-dimycolate) emulsified in mineral oil. Even at a time when the tumor spread microscopically to regional lymph nodes, intralesional BCG not only caused regression of the treated nodule but also cured approximately 50–60% of the animals. Systemic immunity appeared in these cured guinea pigs after regression of the injected nodules had occurred (*16*). Interestingly, systemic resistance to the tumor was not induced by such common methods as the injection of killed tumor cells alone or excision of a primary nodule. To measure the systemic consequences of intralesionally administered BCG further, Zbar and co-workers (*12, 16, 18*) injected a mixture of hepatoma cells and living BCG intradermally and measured the ability of guinea pigs to reject various doses of tumor cells elsewhere. They were able to achieve immunity that was tumor-specific and systemic (against small num-

bers of tumor cells) by mixing BCG with the tumor, but not by injecting each separately. The systemic effect of intralesional BCG is found only after immunity to the tumor was developed, inasmuch as uninjected contralateral nodules usually do not regress at the same time as the injected tumor.

The efficacy of intralesional BCG or BCG–tumor cell mixtures in producing regression of tumors is far more variable in mice than in the guinea pig. In one study, BCG therapy did not influence the growth of a sarcoma or a carcinoma but cured two-thirds of the mice whose melanomas were injected intralesionally (*19*). Prolonged survival but no cures was produced by intralesional injection into primary MCA-induced tumors in these mice. Hawrylko and Mackaness (*20*) and Hawrylko (*21*) reported that optimal immunization against a mastocytoma occurred when killed tumor cells were injected into a site "prepared" by injection with BCG 10 days before, i.e., prophylaxis, whereas immunotherapy was ineffective. Even in other experiments, when tumor growth was inhibited by immunological adjuvants, the development of systemic immunity in the mouse was inconsistent (*22–25*). Bartlett et al. (*25*) noted that the ratio of BCG to tumor cells, specifically to LSTRA lymphoma in an experimental inoculum (or by extension, in an injected lesion), was a key determinant of whether systemic immunity developed in the mouse. High ratios favored local rejection but gave suboptimal systemic immunization, whereas low ratios favored systemic immunity at the expense of local rejection.

In patients with melanoma, intralesional therapy with BCG is effective in approximately one-half the patients (*26, 27*), but only 15% of uninjected lesions regress, and only those in the immediate vicinity of the injected nodules. Furthermore, metastatic melanoma frequently develops in individuals whose injected nodules regressed completely. Therapy of established tumors with BCG or prophylaxis with BCG-tumor cell mixtures was generally ineffective in hamsters (*28*).

Thus although intralesional therapy is often effective and has provided interesting therapeutic models, even when it is useful, it is not necessarily conducive to systemic immunity. Certainly, intralesional therapy does not depend on systemic immunity. The site of inhibition of a tumor is characterized histologically by an intense chronic type of inflammatory exudate, consisting predominantly of lymphocytes and macrophages (*29–32*). Granuloma formation is common at these sites. Although the inflammatory response to BCG may be necessary for the expression of tumor resistance, it may not be sufficient because other inflammatory agents lack similar effectiveness and may even accelerate tumor growth (*30*). Antigenicity of the adjuvant may be essential for its activity (*33*). Apparently, BCG elicits a profound reaction to itself as an antigen, which leads to inflammation and necrosis that encompasses the foreign (tumor) cells in the vicinity and may lead to a systemic state of resistance. All these activities probably involve the macrophages and lymphocytes in the reactive lesion, with immunity resulting from the sensitization of sufficient memory T-cells.

Because of their potential importance in man, BCG–tumor cell vaccines deserve further attention; most immunotherapy administered to humans has thus far been with BCG alone. These combined vaccines offer the possibility of systemic immunization and a true adjuvant effect of the BCG on immunity to an immunogen, but it is apparent from animal studies that the predictability of their success is uncertain. Baldwin and Pimm (*34*) found that experimental pulmonary metastases from rat hepatoma could be restricted by BCG or mixtures of BCG and tumor cells but that metastases were increased with tumors of weak antigenicity. Another important variable to be considered is the amount of BCG in the vaccine. Bartlett et al. (*35*) demonstrated that immunity to the murine leukemia LSTRA, a Moloney sarcoma virus-induced tumor, was potentiated when 5×10^3 or 5×10^5 viable U of BCG were present in the vaccine. However, a dose of 5×10^7 viable U was ineffective, perhaps because of the induction of suppressor cells at this higher dose, as we will discuss shortly. Bekierkunst (*36*) prepared an effective vaccine for the prevention of guinea pig hepatoma, consisting of live hepatoma cells, killed BCG organisms, cord factor, and mineral oil, which obviates the need for living BCG. In rats and rabbits, BCG–polyoma cell vaccines have produced "unblocking" factor in the serum, which then purportedly counteract the deleterious effects of the blocking factor on CMI in those animals (*37*). Whether mixtures of BCG and tumor cells in slow-release emulsions will prove to be the only effective preventatives, or whether schemes in which pretreatment with BCG is a prominent feature, such as the protocol of Hawrylko and Mackaness (*20*), will be effective, remains to be seen.

Despite evidence of such an effect in animals, BCG has not conclusively prevented the development of cancer in man. Although investigators of 2 epidemiology studies suggested that the incidence of leukemia was decreased in children who had received BCG vaccination for tuberculosis (*38, 39*), controlled trials in which the entrants in the study were randomly assigned failed to confirm a significantly lower incidence (*40, 41*). In fact, in the study of Comstock et al. (*41*), an excess of lymphosarcoma and Hodgkin's disease was found and also a slight decrease in the incidence of leukemia.

Deleterious effects have been observed in the tumor-bearing host, particularly when BCG was given after the tumor was well established. Enhancement of tumor growth in animals, described by several investigators (*6, 7, 42, 43*), has been reported anecdotally in man (*44*). When BCG was given to rats prior to a Morris hepatoma (*45*), BCG cell walls also enhanced tumor growth. Pretreatment with complete Freund's adjuvant has had similar adverse consequences with virus- and carcinogen-induced transplantable tumors (*23, 46*). The explanation for enhancement is uncertain but may involve the stimulation of serum blocking factors, antibody–antigen complexes that antagonize CMI to the tumor in vitro and presumably in vivo as well. Blocking factors were in fact demonstrated with BCG-enhanced polyoma tumors and in a patient with metastatic melanoma after intratumor BCG (*43, 44*). However, the appearance of blocking activity in the serum coincident with metastasis in BCG-treated subjects may be more the consequence of tumor growth than its cause, inasmuch as soluble tumor antigen shed into the circula-

tion could then elevate blocking activity (*47*). Another possible explanation for enhancement by BCG is the induction of suppressor cells, either directly or through immune complexes (*48*). Finally, the related phenomenon of antigenic competition, which involves suppressor T-cells, might be important in the enhancement of some tumors. Several groups have reported that BCG has antigenic similarity to some tumors (*49, 50*). Whether this similarity helps to explain some of BCG's protective and therapeutic properties and its tumor-enhancing effects under certain conditions is a matter for conjecture. Because some solid tumors have been enhanced by BCG, it is important that we recognize treatment with systemic BCG, particularly if gross tumor is present, not only may be futile but also potentially dangerous.

Effects of Antibody Synthesis

One of the earliest known effects of tuberculous infection on immunity was the augmentation of antibody synthesis noted by Lewis and Loomis (*51*) with SRBC in the guinea pig. Three years later, Dienes and Schoenheit (*52*) found increased immunity when ovalbumin or timothy pollen was injected into the granulomatous lesion. Since then, many others have demonstrated the adjuvanticity of BCG or its derivatives on antibody synthesis (*53–56*). Miller et al. (*53*), in a particularly carefully conducted study, found that, if the injection of BCG preceded immunization of mice with SRBC by 14 days, maximal augmentation of immunity was achieved; DNA synthesis and cell proliferation were increased in lymphoid tissue after the administration of BCG and appeared to be correlated with its adjuvant effects. Expansion of the pool of antibody-forming cells, B-cells, is one possible explanation of its adjuvanticity; similar findings by Yashphe and colleagues with MER (*57, 58*) support this. Yet it is uncertain whether the effects on B-cells are direct or are mediated through helper T-cells. The PPD, lipid-free BCG [also termed delipidated mycobacterial cells; (*59*)], and other mycobacterial functions, principally peptidoglycans (*60*), are B-cell mitogens in vitro and may have similar activity in vivo. Nevertheless, the necessity for the presence of T-cells for many substances to act as adjuvants (*61*) and the important modulatory role of the T-cell in antibody production suggest that indirect effects of BCG on antibody formation through the T-cell must be considered. Among the many soluble factors released by T-cells is one that stimulates antibody synthesis by B-cells. This material was elicited by re-exposure to T-cells, previously sensitized to BCG in vivo, to the same adjuvant (more precisely, PPD) in vitro (*62*). The specific immune response of memory T-cells reacting to BCG as an antigen led to nonspecific immunostimulation of antibodies to SRBC, through the action of the mediator. Expansion of the pool of helper T-cells, in addition to the pool of B-cells, is another likely effect, which is supported by the findings of Kitamura and associates (*63*). These workers found that living BCG greatly enhanced the response to hamster RBC in SL mice, whose degree of immunity depends sensitively on helper T-cell function. Moreover, BCG stimulation obviated the need for carrier priming in the response of these mice to the hapten trinitrophenyl, which again suggested the recruitment of helper T-cells. Jacobs and co-workers (*64*), studying unimmunized mice, discovered that MER-treated mice had more theta antigen-positive cells binding SRBC than did controls, direct evidence that helper T-cells reactive with antigen were increased by this BCG derivative. The mitogenic effects of BCG on T-cells in vitro (*65*), with a probable counterpart in vivo (*66*), may well underlie the expansion and recruitment of these helper T-cells. However, because some investigators failed to observe mitogenic effects of BCG or MER on T-cells (*67, 68*), further confirmation must be sought before this explanation can be considered plausible.

At certain dosage schedules BCG has also had immunosuppressive effects on antibody synthesis. Those schedules of BCG that favor delayed hypersensitivity tend to depress antibody synthesis (*69*). Repeated scarification of guinea pigs with BCG augmented delayed sensitivity to SRBC but decreased antibody formation (*70*). Our unpublished observations on hemagglutinin titers to SRBC in mice confirm these data. Mice given 1×10^7 viable BCG ip 14 days and 4 days before SRBC had hemagglutinin titers of 1:2, compared with 1:16 for controls without BCG. The BCG failed to augment cytotoxic antibody titers against H-2d alloantigens when given 10 days before irradiated H-2d (L1210) tumor cells, a schedule that augmented CMI to the same antigens (*71*). Whether suppressor cells of the T-cell or macrophage series were stimulated by BCG, accounting for its failure to augment immunity, was not explored in these experiments. Such a mechanism is possible because suppressor cells, especially macrophages, can be induced by BCG (*66, 72, 73*).

Effects of Cell-mediated Immunity

As previously mentioned, the adjuvant effect of an active tuberculous infection on CMI was first noted by Dienes and Schoenheit (*52*), who determined that increased DTH reactions to ovalbumin and timothy pollen were induced by injecting these soluble antigens into a tuberculous lesion. Freund's complete adjuvant augments delayed hypersensitivity to soluble antigens because of its content of killed mycobacteria (*74*). Either BCG or its derivatives can potentiate CMI to protein antigens (*75*), to haptens conjugated with normally nonimmunogenic carrier molecules (*76*), and to poorly immunogenic tumor lines (*21*). The wax D fractions from several human types of *Mycobacterium tuberculosis* could mimic the adjuvant effect of the whole killed mycobacteria, including the production of corneal hypersensitivity, when injected with a protein antigen in a water-in-oil emulsion. However, wax D from BCG apparently lacks a critical peptide component, which causes it to be inactive (*77*).

The survival of skin grafts is profoundly affected by the administration of BCG (*70, 78–80*). Accelerated rejection of these skin grafts usually has been produced by pretreatment with BCG, particularly with allografts. However, Zschiesche and Heinecke (*80*) found that syngeneic male-to-female skin grafts were rejected in an accelerated fashion if BCG were administered 21 or 28 days before grafting, but prolongation of graft survival was produced by BCG given twice, 7 and 36 days before grafting. This prolonga-

tion was not observed with allogeneic skin grafts, whereas the accelerated rejection was also demonstrable with allografts. Significantly, mice that received BCG twice had a more pronounced stimulation of the phagocytic activity of their RES than those that received BCG once and yet had prolonged graft survival. A likely explanation for this paradox is that suppressor macrophages were induced concomitantly with killer and phagocytic macrophages.

The survival of solid spleen allografts and isografts was decreased markedly by BCG, whereas dispersed syngeneic or allogeneic spleen cells survived normally in vivo despite pretreatment of recipients with BCG (*81*). That the initial engraftment was unaffected suggested that BCG increased the proliferation of immunocompetent lymphocytes following recognition of antigen. Nonspecific RES stimulation does not seem to be of primary importance in the accelerated rejection of allografts and isografts produced by BCG and its derivatives (*82*), inasmuch as the maximal resistance to isografts induced by MER occurs after RES activity has returned to normal. In addition, RES stimulation has been associated with decreased rapidity of rejection (*80*), not necessarily accelerated rejection. Splenectomy alone between BCG administration and skin grafting abolishes the accelerated rejection (*78*), but this does not provide conclusive evidence for or against the importance of the RES.

Antagonism of Immunosuppression

Among the most interesting and potentially exploitable effects of BCG and its derivatives, particularly MER, is their ability to reverse or protect against the immunosuppressive effects of various agents. The MER caused nearly complete reversal of the immunosuppressive effects of antithymocyte serum on the circulating antibody response to SRBC, whether given before or after the serum (*83*). In addition, MER protected against the suppressive effects of cortisone acetate on the appearance of rosette-forming cells after immunization with SRBC. The cells, as measured by spleen cell-mediated cytotoxicity against [^{51}Cr]-labeled targets, were similarly affected (*84*).

Pretreatment with BCG increased the number of PFC directed against SRBC and could partially protect against or reverse the immunosuppressive effect of MCA (*85*). The combination of BCG and MCA resulted in approximately the same number of antibody-producing cells as in immunized controls given neither agent. Pretreatment with BCG also conferred long-lived protection against the immunosuppressive effects of the antimetabolite ara-C. Spleen CMI to allogeneic L1210 cells was significantly decreased by the 20 mg ara-C/kg per day for 5 days but was the same as in immunized controls in the group also given BCG iv 10 days before the antigen (*71, 86, 87*). Treatment with BCG after chemotherapy caused a more transient but significant reversal of immunosuppression. Investigation into the type of lymphoid cell affected by ara-C and BCG thus far suggests that the same cell diminished by the one is increased by the other and that it is a member of the monocytic series (*see* discussion under Effects on Macrophages). In our studies, cytotoxic antibody production and T-CMI were unaffected by BCG pretreatment, which failed to antagonize their immunosuppression by ara-C.

Pretreatment with BCG apparently protected against the immunosuppressive effects of the alkylating agent CPP because mice could be made resistant to the induction of a tumor by Moloney sarcoma virus or could be immunized against challenge by a Moloney sarcoma virus-induced tumor even in the face of chemotherapy with CPP (*88, 89*). However, inasmuch as specific in vitro measurement of immunity was not made in these studies, these effects may have been partly due to the induction of IF. Yet further support for an immunostimulatory antagonism of immunosuppression comes from studies of Mathé and colleagues (*5*) on immunity to leukemia L1210. The therapeutic benefit from high toxic doses of CPP against syngeneic L1210 leukemia in mice could be increased by subsequent treatment with BCG. The increased efficacy of combined therapy was attributed by these authors (*5*) to a myeloprotective effect of BCG but could just as well have resulted from reversal of immunosuppression. In contrast to studies with Moloney sarcoma virus and allogeneic L1210, pretreatment with BCG appeared to increase the number of drug-related deaths attributable to CPP. Whether this effect was ascribable to the induction of DNA synthesis in myeloid or lymphoid cells, thereby increasing their susceptibility to the cytotoxic effects of CPP, was not established.

Reversal of immunosuppression was also found in antigen- and antibody-induced suppression of macrophage receptors for cytophilic antibody (*90*). In this system, peritoneal macrophages from mice treated 10 days previously with specific immune complexes were unable to attach cytophilic antibody to leukemia L1210 in vitro. This suppression can be prevented or reversed by the injection of viable BCG 5 days before or after suppressive treatment (Rao VS, Grodzicki RL, Mitchell MS: Unpublished data). Because suppressor T-cells are intimately involved in this phenomenon (*48, 91*), it is not clear whether the protection and reversal effected by BCG are mediated through T-cells or are direct effects on the macrophage membrane. Direct effects of BCG on the membrane may influence the resistance of spleen cells to immunosuppression by antilymphocyte serum; isolated cells treated with MER in vitro were more resistant to cytolysis in vitro by antilymphocyte serum and complement (*84*).

Stimulation of Effector Macrophages

In its activity as a protective vaccination against *M. tuberculosis,* BCG presumably acts to increase specific CMI to that organism, with which it shares antigenic cross-reactivity (*92*). However, in addition, alveolar macrophages from rabbits given injections of BCG show an increased ability to inhibit nonspecifically the growth of intracellular organisms other than mycobacteria, with an accompanying increase in the content of lysosomal hydrolases (*93*). Nonspecific cytotoxicity to unrelated bacteria can also be induced in macrophages from animals immunized with *M. tuberculosis, Listeria monocytogenes, Brucella abortus,* or *Toxoplasma gondii* (*24*). With BCG, the rate and onset and the strength of cytotoxicity depend

on the vaccination dose and require the development of a delayed sensitivity reaction to BCG itself. Heat-killed BCG are ineffective in the induction of nonspecific antimicrobial cytotoxicity; chronic injection with living bacteria is required. Alternatively, the persistence of mycobacteria can be accomplished by the injection of killed organisms in complete Freund's adjuvant (*24*). Protection against *M. tuberculosis* can be achieved by nonviable BCG, with BCG cell walls attached to oil droplets, or a combination of peptidoglycolipid CWS attached to the lipid component (*94*).

The random migration, chemotaxis, and pinocytosis of macrophages in the guinea pig (*95*) is increased by BCG as is chemotaxis in the macrophages of the mouse (*96*). Chemotaxis effected by several strains of BCG was elevated, beginning 4 days after the mycobacteria and lasting through day 21. Pinocytic capacity was increased only briefly during the third and fourth weeks after inoculation (*95*).

Mice inoculated with BCG have an accelerated rate of clearance of *E. coli* endotoxin and of carbon particles by macrophages of the RES (*97*). Animals thus inoculated also clear *Salmonella enteritidis* more rapidly than do control animals and catabolize denatured albumin more rapidly; BCG also protects against *Staphylococcus aureus* and *Candida albicans* even in immunosuppressed mice, which suggests that BCG-stimulated macrophages are involved (*98*).

Although the protective effect against microbes is wide-ranging, i.e., from bacteria to viruses, BCG does not cause macrophage-mediated protection against all organisms. Macrophages activated by BCG capable of destroying *Listeria* cannot kill *Yersinia tularensis* (*99*). Furthermore, even when BCG is protective, the mechanism may not involve the macrophage. Pretreatment with BCG completely prevented the infection usually caused in CBA mice by *Babesia* and *Plasmodium* (*100*). Yet these protozoa were killed intraerythrocytically after their appearance in the peritoneum, and immunity was long-lived; mediators released from immunized T-cells may have been involved. Salvin et al. (*101*) have in fact found substances in the sera of BCG-infected mice with IF and MIA after restimulation with BCG, tuberculin, or certain unrelated antigens. Moreover, BCG protects against the local growth and metastasis of *Echinococcus* hydatid cysts (*102*), in much the same manner as against metastatic tumor cells (*34*), but has no effect against *Treponema pallidum* infection (*103*). Thus, even though stimulation of CMI may be involved in the effects of BCG, that does not necessarily mean that macrophage-mediated killing is involved in all instances, nor that the stimulation of CMI will lead to nonspecific protection against all organisms.

Hibbs et al. (*104–106*), Cleveland and associates (*107*), and Holtermann and his co-workers (*108*) have shown that BCG-activated macrophages are capable of recognizing and destroying various neoplastic cells. With few exceptions (*109*), these activated macrophages can discriminate between neoplastic and non-neoplastic (usually nontransformed fibroblast) cells. In general, early passages of embryo cells, which show little contact inhibition, are susceptible to killing BCG-activated macrophages, whereas later passages are minimally affected (*107, 108*). Peritoneal macrophages induced by substances such as starch, mineral oil, or thioglycolate were not nonspecifically tumoricidal, even though these frequently appeared morphologically activated. This is an in vitro correlation of the earlier observations that activation of macrophages is necessary but insufficient for the expression of the tumoricidal effects of BCG. The manner in which BCG-activated macrophages discriminate between tumor cells and normal cells is uncertain; whether discrimination involves recognition of the antigenic structure or another characteristic, such as the proliferative rate of the neoplastic cells, has not been conclusively established. Evans and Alexander (*110*) have shown that specific recognition of tumor antigens on a syngeneic tumor by macrophages in tumor-immunized mice can be promoted through an arming factor from T-cells, but macrophages nonspecifically activated by BCG, lacking the specific arming factor, kill various tumor cells. Moreover, cytophilic antibody derived from B-cells constitutes another such arming factor (*111*). Thus a relative discrimination is possible in BCG-activated macrophages and to a much greater degree in armed, activated macrophages.

When BCG is injected iv into mice, splenic mononuclear cells that may be monocytes (macrophages) are stimulated to become cytotoxic to tumor cells even if they have not previously been exposed to the tumor (*66*). In our original experiments, BCG augmented CMI to P-815Y mastocytoma cells (measured by a 48-hr microcytoxicity assay) when given with suboptimal doses of tumor cells to allogeneic mice (*66, 112*). Controls given BCG alone, however, had the same degree of CMI as those given BCG and alloantigen. The pseudoimmune mononuclear cells involved in CMI were at first assumed to be T-cells because they were not significantly reduced by adherence to glass or plastic surfaces and T-cells were known to be effector cells in a 6-hour ^{51}Cr release assay with the same alloantigenic system (*113*). However, further work has shown that these mononuclear cells are not sensitive to anti-theta serum, are developed even in thymus-deprived mice, and have a range of killing (several types of tumor cells but not normal fibroblasts) that suggest they are in the monocytic series (Kinder BK, Mitchell MS: Unpublished data). They are detectable only by a 48-hour growth inhibition assay, not by a 6-hour ^{51}Cr release assay. We have found that similar cells are elicited by BCG given to mice 10 days before an alloantigen. The major difference is the specificity of the cytotoxicity these cells display toward the immunogen, when alloantigenic (L1210) cells are given in conjunction with BCG (*71, 86, 87*). The mononuclear cells produced by BCG and alloantigens are insensitive to anti-theta serum, undetectable by 6-hour ^{51}Cr release, unaffected by glass and plastic adherence, and resistant to 500 and 1,000 rad in vitro radiation. Evidence for surface immunoglobulin was equivocal. Nylon wool columns trap some of the cells, but many others pass through, and even the adherent cells are readily elutable. Perhaps the most direct evidence that the cells are not T-cells but depend on T-cells for their specificity comes from experiments in which the cells were partially depleted by treatment with carbonyl iron and a magnet. Repletion of the cells removed by this procedure

could be accomplished with purified macrophages from the peritoneum of normal mice. Treatment of the spleen cells with anti-theta serum prevented repletion by normal peritoneal cells, whereas anti-theta treatment of the peritoneal cells had no effect. Inasmuch as the splenic effector cells were not killed by silica but were depleted by carbonyl iron magnet treatment, they were most probably not phagocytic but rather adherent to the outside of the carbonyl iron particle. The weight of evidence thus far suggests that the cell induced by BCG and alloantigen is of the monocytic series, armed specifically by a T-cell factor.

Kiessling et al. (*114*) and Herberman and co-workers (*115*) described nonadherent NK cells in the peritoneal exudate of mice, which cause a low background level of cytotoxicity against foreign cells in unimmunized mice. The NK cell lacks the markers characteristic of classical T- and B-cells and is also not a mature macrophage because it is nonadherent and nonphagocytic. Trypsinization markedly decreases its function, which is also labile at 37° C. However, BCG, but not proteose peptone or thioglycolate broth, can increase the activity of peritoneal cells with similar characteristics (*116*), although cells stimulated by BCG cannot be called "natural," strictly speaking; the term applies to an ostensibly uninduced population. The peritoneal cells used by Wolfe et al. (*116*) were cytotoxic in a 4-hour ^{51}Cr release assay and thus are different from the nonadherent, nonphagocytic inhibitory spleen cells evoked by BCG and the alloantigen described above. Yet the nonadherent, nonphagocytic peritoneal NK cell may well be related to the nonadherent, nonphagocytic splenic mononuclear cell, with both representing varieties of immature macrophages lacking many of the features of the fully developed cell. Peritoneal adherent cells capable of killing allografted tumor cells in a 16-hour ^{51}Cr release assay were increased by treatment with ip BCG, as well as other adjuvant substances (*117*). These authors did not test the specificity of these cells after the administration of antigen and adjuvant, thereby making it difficult to relate them further to splenic mononuclear effector cells. Nathan et al. (*118, 119*) described a related peritoneal nonphagocytic adherent cell that was cytotoxic to tumor cells, induced by BCG alone. They believed that this subpopulation of peritoneal cells is not a variety of macrophage but rather a peculiar B-cell; however, none of their data completely rule out the former possibility. The same group had previously reported that several tumor lines were enhanced in their growth by normal peritoneal cells in vitro but less enhanced by cells in BCG-treated mice (*120*).

The low level of nonspecific background inhibitory activity against tumor cells found in the peritoneum or spleen of the mouse may simply reflect the few killer cells present, which may be natural or induced by the normal microflora impinging upon the animals. Expansion by BCG or other adjuvants of this subpopulation of inhibitory cells thus may lead to a population that is grossly cytotoxic to tumor cells rather than stimulatory.

Stimulation of Afferent Macrophages

Also influenced by BCG are macrophages involved in the afferent arm of immunity. Lymphocyte-activating factor, also known as IL-1, a substance that causes proliferation of T-cells in mice (*121, 122*), is produced by such macrophages in the peripheral blood, peritoneum, or spleen of several species of animals. The spleen of a BCG-treated mouse produces twofold to threefold more of this factor than a comparable normal spleen (*66*), in proportion to the increased percentage of macrophages. Purified adherent cells from the BCG-treated spleen produce increased amounts of IL-1, again in proportion to their increased number. Treatment of splenic cells with BCG in vitro leads to a twentyfold increase in the amount of factor produced (*65*), whereas various derivatives of BCG, such as MER and lipid-free mycobacteria, stimulate splenic macrophages threefold to sixfold above base line in vitro (*59*). The IL-1 may be responsible for the mitogenic effect of BCG on T-cells, acting as an intermediary stimulator and expander of the number of reactive T-cells. Some investigators have postulated that the profound amplification of cellular immunity produced by BCG may depend on such proliferation-inducing mediators, produced not only by macrophages but by T-cells as well (*59, 121*). Yashphe (*58*) and Weiss (*83*) have also obtained some evidence that MER acts to improve the processing or presentation of antigen (SRBC) by macrophages. By analogy with other adjuvants, such as *Bordetella pertussis* and *E. coli* LPS, which clearly augment antibody production in part through stimulation of antigen-laden macrophages (*123, 124*), BCG and its derivatives may improve the presentation of antigen in more immunogenic form or may alter the macrophage membrane to permit more avid association with antibody-producing (B) cells.

Induction of Suppressor Adherent Cells

In addition to its stimulation of afferent and effector macrophages, BCG can elicit suppressor adherent cells that are most likely macrophages. These suppressor adherent cells induced in BCG-treated mice inhibit the response to the mitogens Con A and PHA by splenic T-cells (*66, 72*). Their partial depletion by adherence to glass led to improvement in the response to both mitogens in normal mice and especially in BCG-treated mice in which their suppressive activity was greatly increased. The effect on the response of spleen cells to B-cell mitogens, such as LPS, could not be measured accurately because sticky B-cells were also removed by glass adherence; BCG is itself a mitogen for splenic T-cells (*36, 65*). The response to BCG of normal spleen cells was improved by removal of adherent cells by serial adsorption to plastic, which reduced their percentage from 10 to 1 (*65*). In addition to the evidence that a small percentage of macrophages is required for BCG to stimulate T-cells (*65*) and the complementary evidence of Fölch et al. (*125*) on the optimal percentage of macrophages needed to permit a response to PHA (1–5%), these data indicate that a concentration of 5–10% of macrophages (the normal range) may in fact be supra-optimal and thereby suppressive. By increasing both the number and activation of macrophages, BCG may inhibit mitogenic responses.

Direct evidence for adherent suppressor cells activated in the spleen by BCG has come from independent coincidental investigations of Bennett, Mitchell and co-workers

(*73, 126, 127*), and Klimpel and Henney (*128*). These scientists determined that in vitro immunization of C57BL/6 mouse spleen cells by allogeneic P-815Y tumor cells was abrogated by a large dose of BCG administered approximately 1 to 6 weeks earlier. They noted 2×10^7 viable U of BCG or more iv attracted natural suppressor cells, i.e., cells were present in normal bone marrow, from the bone marrow to the spleen, where they were activated by the BCG organisms and where they proliferated during the second week after BCG (*73, 126*). Only nylon-adherent, anti-Thy 1-resistant spleen cells from BCG-treated spleens were effective suppressors. Admixture of these cells with normal spleen cells, at a ratio as low as 1:10, significantly inhibited the ability of the normal cells to be immunized by the alloantigeneic tumor cells. These adherent suppressor cells, which seem to be a variety of macrophages (*127, 128*), block the development of cytotoxic T-cells and non-T-killer cells, most likely by inhibiting proliferation. The same mechanism would explain antagonism of the response to mitogens. The nonadherent cells from BCG-treated spleen had the capacity to be immunized once removed from the presence of the adherent cells. Furthermore, if cytotoxic cells were already generated, the adherent cells had no effect on their activity. Specifically, both the addition of adherent suppressor cells to sensitized T-cells and the removal of adherent cells once immunization was achieved failed to influence the cytotoxicity against the target cells (*73*). These suppressor cells release prostaglandins and can be inhibited by prostaglandin synthetase antagonists (*127*).

A collaborative attempt by scientists at the two laboratories involved to reconcile the experiments of Braun et al. (*129*) on the augmentation of anamnestic immunization in vitro by BCG with those of Bennett and associates (*73*) on suppressor macrophage induction led to the discovery of 2 strains of BCG that differ remarkably in their ability to induce the suppressor cells. Braun et al. (*129*) found that with the mineral oil-induced plasmacytoma MOPC-315, BALB/c mice given Phipps BCG ip while bearing the primary tumor had spleen cells that were enhanced in their ability to respond to a rechallenge with MOPC-315 cells in vitro. The collaborative study of several batches of this strain of BCG in comparison with the Tice BCG used by Bennett and colleagues showed that Phipps strains 734 and 724 had purely augmentative effects when given in vivo, whereas Phipps strain A-8 and Tice BCG induced suppressor cells. These effects were demonstrable in both the syngeneic (MOPC–BALB/c) and allogeneic (P-815Y–C57BL/6) systems (*130*).

The adjuvanticity usually seen in vivo after administration of BCG is probably always the vector sum of several opposing forces, of which the negative vector, induction of suppressor adherent cells, is one. The strength of immunogenicity of the antigen, and, more importantly, the timing, dose, and strain of BCG are certainly other factors one should consider in predictably influencing the outcome of immodulation. The concomitant presence of macrophage-like killer cells in the same spleen that contains suppressor cells indicates that several types of macrophage activity are elicited. There may well be distinct subpopulations of macrophages akin to those found with lymphocytes: afferent (helper), effector (cytotoxic), and suppressor, perhaps with identifying phenotypic markers analogous with the Ly antigens. The alternative, that different types of assays measure different properties of the same population of macrophages, has not been ruled out, however. This issue of subgroups of macrophages is considered in more detail in another review (*131*).

Alteration of Lymphocyte Trapping

The concentration or "trapping" of PBL in the spleen and lymph node can be altered by BCG. Zatz (*132*) found that splenic trapping of [^{51}Cr]-lymphocytes in the mouse was increased briefly in the spleen 1–2 days after BCG, then again 3 weeks later, whereas in lymph nodes trapping was persistent. Either iv or sc administered BCG increased trapping in lymph nodes, but iv BCG decreased spleen trapping. The macrophage has been implicated in this process of concentrating lymphocytes by BCG (*133*), although Zatz (*132*) believes that T-cells also are involved. Whether suppressor macrophages or suppressor T-cells might be involved in the negative trapping in the spleen is a matter for further study. Whole viable BCG cells are not required to augment trapping. With techniques similar to those of Zatz (*132*), Ogura et al. (*134*) showed in the tumor-bearing rat that BCG cell walls attached to oil droplets increased the influx of lymphocytes into the regional lymph node to a greater extent than that induced by the AH-130 hepatoma alone.

Effects on T-cells

As mentioned previously, BCG is mitogenic for T-cells (*65*), and it stimulates not only splenic nonadherent cells enriched for T-cells but also T-cells in the Swiss CD-1 and inbred C3H mouse. A critical concentration of macrophages of approximately 0.25–0.5% is required for this effect, inasmuch as removal of macrophages (below 0.25%) abolishes the mitogenic effect of BCG, and reconstitution to 0.5% restores it (*65*). Thus the effect on T-cells may be indirect through the macrophage, which is similar to findings on the mitogenicity of PHA (*135*). Bekierkunst (*67*) was, for reasons not immediately apparent, unable to stimulate thymic T-cells with BCG unless they were admixed with spleen cells; MER stimulated T-cells in vitro, but delipidated mycobacterial cells failed to do so (*90*), which perhaps indicated a requirement for lipid. The dose–response curves described for spleen and T-cells with BCG resemble those for other T-cell mitogens, such as Con A, with inhibitory effects at the highest concentration (250×10^5 viable U/ml) and a sharp optimum at approximately 1×10^6 viable U/ml.

The relationship of the mitogenicity or antigenicity of adjuvants and their activity as adjuvants is not entirely clear, but it appears that many adjuvant substances are either mitogens or antigens (*65, 136*). Specific desensitization to BCG abolished its intratumoral effect (*13*). Moreover, the strains of BCG that were most antigenic were also the strongest adjuvants in vivo (*35*). Many, but not all, peptidoglycans isolated from mycobacteria that have adjuvanticity have been mitogenic, particularly B-cell mitogens acting on spleen cells from the nude nouse (*60*).

Some strong mitogens, such as Con A and PHA, have predominantly immunosuppressive effects in vivo (*137*) or in vitro (*138*), which have been due to the production of suppressor T-cells in at least one circumstance (*138*). Mitogenicity for lymphocytes did not always correlate with the production of specific lymphocyte-made mediators or the development of cytotoxic effector cells (*139–142*), but the incorporation of [^{3}H]dThd into the DNA of lymphocytes may constitute a simple means by which rapid screening of substances for possible adjuvanticity can be achieved.

Although our early experiments with BCG in vivo were interpreted as showing improved spleen T-cell-mediated cytotoxicity against allogeneic tumor cells, it is unproved that the effector cell does not kill within 4–6 hours and is insensitive to anti-theta serum, making it unlikely that it is a T-cell. Measured by ^{51}Cr release within 6 hours, cell-mediated cytotoxicity was unchanged in mice given 1×10^7 BCG 10 days before the alloantigen, when tested 14 days after the antigen (*71*). Pretreatment with complete Freund's adjuvant virtually abolished the cytotoxic T-cell response to alloantigens in mice, although the memory function of T-cells was unaltered (*143*). Perhaps scheduling is once again of considerable importance in the determination of whether BCG affects the function of T-cells. Braun et al. (*129*) augmented the in vitro response of mouse splenic T-cells to syngeneic tumor cells by pretreatment with BCG in vivo 11 days before the start of incubation. It is also likely that many of the effects of BCG on allografts and tumors in vivo depend on the augmented proliferation of sensitized T-cells, as judged from extensive histological observations (*13, 29, 33*), even though the direct demonstration of increased number and activity of T-cells by in vitro assays has not always been possible.

Priming of Mice for Production of Tumor Necrosis Factor

A glycoprotein of 150,000 daltons, TNF is released into the serum of mice pretreated with iv BCG 2 weeks before iv stimulation of *E. coli* LPS (*144, 145*). As its name states, this material causes hemorrhagic necrosis of several transplantable mouse tumors in vivo or in culture, but normal embryo fibroblasts are unaffected. A BCG pretreatment is essential for endotoxin challenge to be effective in eliciting TNF. However, other agents causing reticuloendothelial proliferation, such as *C. parvum* or zymosan, can substitute for BCG. Although the origin of TNF is undetermined, it may come from the activated macrophages present in abundance in the spleen of BCG-primed animals. Because the spectrum of activity of TNF resembles that of BCG-activated macrophages, sparing nontransformed cells, TNF may be one of the soluble mediators released during the destruction of tumor cells, which, under ordinary circumstances, are not found in the serum but only in the immediate vicinity of the rejection reaction. The physiological significance of TNF release is open to question, particularly because the mice producing the substance are frequently moribund of endotoxin shock, but its potential efficacy as passive immunotherapy of tumor-bearing mice is a matter of considerable interest.

Effects on Interferon, Colony-stimulating Factor, and Pyrogens

Although IF and CSF are not really immunological mediators, their stimulation represents paraimmunological effects of immunological modulators that may also be exploitable therapeutically. These substances appear to be members of a family of macrophage-made materials, of which lymphocyte-activating factor is another example. Whereas BCG can induce IF activity (*101*), which is made by both lymphocytes and macrophages, this property is shared by many other immunological modulators (*146*). In fact, agents first identified as inducers of IF, such as poly I:C and poly A:U, and the fluorenone derivatives have many properties of adjuvants, which suggests that each class of agent be investigated routinely for the other property. Whether any additional therapeutic gain will be realized from antitumor therapy with BCG against tumors by oncogenic viruses remains to be seen.

The CSF, which stimulates the growth of myeloid colonies from the bone marrow in agar culture, has been increased by BCG in the dog (Kelly M, Marsh JC, Mitchell MS: Unpublished data), and by the adjuvants LPS and *C. parvum* in the mouse (*147, 148*). It is likely that other such trophic and regulatory substances are produced by macrophages and that stimulators of macrophages, such as BCG and its fractions, will prove useful as augmentors of their production. Antagonism of the myelosuppressive effects of chemotherapy is one such use. An increase in suppressor substances from macrophages would also be predicted by this reasoning and from the known effects on BCG in eliciting suppressor macrophages.

Pyrogenic substances produced by lymphocytes represent another paraimmunological response, which often accompanies immunity and can be induced by immune complexes among other stimuli (*149*). Not only is BCG pyrogenic, like many similar organisms, but there appears to be a general correlation between the adjuvanticity of components of bacterial cell walls and their pyrogenic effects (*150*). However, if the two properties prove inextricably linked, this would obviously be a hindrance to the isolation of nontoxic components with immunomodulatory capacity.

Fractions of BCG

A number of derivatives of BCG have been produced in an attempt to find fractions that share the beneficial attributes of BCG but lack its toxicity. We will exclude MER from this discussion because it has been reviewed in detail elsewhere (*68*). Lipid-free derivatives prepared from BCG or related mycobacteria have been capable of protecting against several ascites leukemias and Ehrlich ascites carcinoma in mice (*6, 151, 152*). Although lipid-free mycobacterial cell walls and lipid-depleted cell walls (also known as CWS) are effective against dispersed tumors, the effectiveness of fractions against solid tumors, particularly by the intratumoral route, appears to depend on the additional presence of lipid. Zbar et al. (*13*) and Ribi and co-workers (*153*) physically reattached cell walls and CWS to oil droplets and found their activity restored to that of whole BCG against solid tumors. Not only are BCG cell walls in saline ineffective, but pretreatment with them

creates an unresponsive state to cell walls in oil, which abrogates the latter's specific antituberculous protection, minimizes granuloma formation, and causes a loss of footpad swelling on challenge with PPD (*154*). Lipid-free CWS have afforded some protection against tumors when mixed with autologous mammary tumor cells and reinjected, but systemic immunity was not produced because new mammary tumors arose despite rejection of the primary (*155*).

The contribution of lipid to the therapeutic effects of BCG may be its ability to elicit granulomas, both nonallergically and immunologically determined. Highly refined fractions of BCG are apparently incapable of evoking a strong inflammatory response. Although that may lessen their toxicity, it is also a therapeutic drawback; BCG cell walls attached to mineral oil increase the trapping of lymphocytes into a lymph node draining the site of a tumor to a greater extent than that caused by the tumor alone (*133*). Tumor growth and metastasis to the lymph node were both suppressed in those experiments. The granuloma formation caused or abetted by lipid may depend on stimulation of T-cells, with their subsequent elaboration of soluble mediators. Some evidence shows the need for lipid in the stimulation of T-cells from experiments on the mitogenicity of BCG and its lipid-free fraction, delipidated mycobacterial cells, which was incapable of stimulating T-cells in contrast to BCG, a strong T-cell mitogen (*59*). Ineffectiveness against solid tumors of lipid-poor fractions might be an in vivo reflection of their inability to stimulate T-cells. The rejection of dispersed tumors and leukemias in mice seems to depend more critically on antibody and non-T-cells [(*156*); Deegan S, Mitchell MS: Unpublished data]. Therefore, cell walls, as water-soluble, lipid-free supernatant fractions of sonicated BCG plus lipid-depleted organisms, which are all stimulants of splenic B-cells (*59, 157*), would be expected to have activity against them. Yamamura et al. (*158*) significantly increased specific T-cell-mediated cytotoxicity to a suboptimal dose of P-815-X2 mastocytoma cells by the administration of lipid-free CWS and mineral oil. Background cytotoxicity with P-815-X2 alone, by ^{51}Cr release, was 15%, whereas CWS–oil-treated mice had 66–100% lysis. The contribution of oil droplets in the induction of T-cell-mediated lysis was considerable, as oil alone (with P-815-X2 cells) improved lysis to a level of 48–84%. Although the effect was clearly augmentative on T-cells with admixing CWS with the oil, the authors unfortunately did not test what effect the skeletons by themselves had on immunity. Thus the lipid intrinsic to BCG has effects that were mimicked and thus elucidated by admixture with oil.

Ribi et al. (*153*) diagrammed the structure of the CWS, which exists as an insoluble polymer, a peptidoglycolipid, as shown in figure 5-1. It appears that one component lost in the removal of free lipids from BCG or its cell wall is the trehalose-mycolic acid ester, trehalose-6,6′-dimycolate, known as "cord factor" (*159*) or as "P_3" (*153*). This mycolate component appears to be capable of including granulomata by itself, although these are more transient than with the whole organism and are ineffective at causing complete regression. The intratumoral injection of P_3 added back to CWS in oil caused tumors that were unresponsive to the skeletons alone to be rejected successfully (*153, 154*). Even when BCG is simply killed and lyophilized, cord factor may be lost because the addition of this factor to such BCG in oil or aqueous medium restored its capability as an antitumor agent in intratumoral therapy and as a vaccine (*17, 36*).

Various combinations of P_3 with other bacterial materials have been examined for antitumor properties when injected intralesionally. With the guinea pig line-10 hepatoma, Ribi and co-workers found that a combination of P_3 and a glycolipid from *Salmonella* Re mutant, particularly when given as an emulsion, were particularly effective in causing regression of the tumor. Re glycolipid with CWS of BCG were also effective and caused nearly 100% regressions if either of them was given in an oil–water emulsion (Ribi EE: Unpublished data). In these studies, large amounts of material (150 to 350 g) have been injected exclusively intratumorally, so that it is difficult to be certain whether the tumor would have been killed and that there would have been a persistent, long-lived systemic immunity generated if the materials had been given systemically instead. The amounts injected, 150 to 750 μg, represent more than the amounts of the components contained in the whole BCG usually injected. Also, most of the tumors tested have been immunogenic. Whether any of these components, cell wall, CWS, or P_3, can boost the specific immunity to the TAA has not been conclusively tested, although it is likely that the peptidoglycan-containing portions are true adjuvants. Endotoxin contamination and the endotoxin properties of some of the fractions, as well as the Re glycolipid, may also prove to be problems in translating these results to the clinical situation.

The component responsible for the adjuvants of BCG

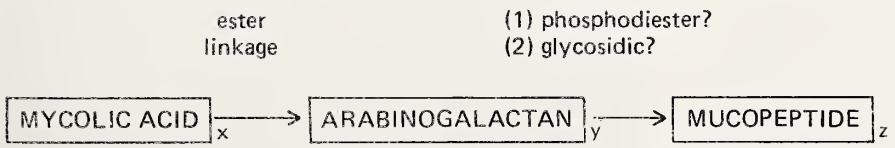

FIGURE 5-1.—Structure of the CWS, a peptidoglycolipid. *See* (*153*).

C_{76}-C_{84}
a-C_{24} branch
b-OH

D-Arabinose
D-Galactose

N-acetyl-glucosamine
N-glycolyl-muramic acid
Alanine
Glutamic acid
Diaminopimelic acid

may be a small peptide present in the peptidoglycan (mucopeptide) portion of the cell wall. Although mycobacteria were not specifically the subject of their investigation, Fleck et al. (*160*) showed with several gram-positive and -negative bacteria that the smallest bacterial structures containing adjuvant activity were peptides of the general forms:

where R_3 was either a meso-diaminopimelic acid or an N-substituted L-lysine residue. The MDP closely resemble the active portion of mycobacterial peptidoglycan that is largely responsible for its modulatory capacity. Attempts to deduce the limit peptide responsible for adjuvanticity by degradative techniques alone were unsuccessful. Thus the MDP characteristic of the mycobacterial peptidoglycan was actually synthesized rather than obtained analytically, but strong evidence suggests that this synthesized material is in fact identical with the limit peptide. A series of MDP has now been synthesized, with various expansions and substitutions in their structure, principally by Chedid and his collaborators (*161, 162*). These materials are water soluble and active orally. Synthetic MDP analogous with material (*161, 162*) from bacterial cell walls, including mycobacteria, have had adjuvant effects on antibody synthesis and delayed sensitivity (*150*) and have protected nonspecifically against *Klebsiella* infection, possibly through macrophages (*161, 162*). These MDP are closely related to the peptides studied by Fleck et al. (*160*). One strongly adjuvant MDP with the structure *N*-acetylmuramyl-L-Ala-D-Gly-NH_2 is probably the minimal natural peptide in BCG.

Interestingly, MDP with D-alanine instead of the natural L-isomer depress the immune response rather than augment it (*161*). Both the CWS with its peptidoglycan and P_3 appear to be necessary for the effective intratumoral therapy of guinea pig hepatoma and long-lived subsequent systemic protection (*153*).

The series of MDP developed by Chedid and his collaborators have greatly expanded our understanding of the chemical basis for the adjuvanticity of BCG. Unlike many derivatives of BCG, MDP can substitute for whole BCG in minuscule amounts, thereby increasing both nonspecific immunity (through macrophages) and specific immunity when given with antigen. They can also stimulate macrophages to release prostaglandins, endogenous pyrogen, and lymphocyte-activating factor and can activate macrophages to kill tumor cells in vitro. Longer derivatives in the series are more easily phagocytized and hence are the most effective in stimulating macrophages. Cytolytic T-cells specific for an antigenic target are increased after antigen given with MDP. When conjugated to the synthetic carrier T, G, A----L, MDP obviates the need for Freund's adjuvant in several test systems of antibody production. The most exciting aspect of the MDP from a theoretical standpoint is that they offer one the opportunity to manipulate the critical structure responsible for adjuvanticity of mycobacteria and thus to modulate biological activity toward various types of lymphoid cells. One can thereby learn the critical determinants that affect such activity. Thus many that will prove useful clinically will undoubtedly not be the naturally occurring dipeptides of microorganisms but rationally designed derivatives. They appear to have low toxicity in early clinical trials and can be used systemically by a variety of routes.

Thus because BCG is a complex biological material, it is likely that attempts toward isolation of portions possessing specific properties, such as granuloma formation, adjuvanticity, and perhaps even cross-antigenicity with certain tumor cells, will be fruitful in continuation of the development of BCG as a therapeutic agent.

Therapeutic Trials

Several recent concise reviews have dealt specifically with clinical immunotherapy and should be consulted for more extensive information (*163, 164*). Our aim here is to point out a few representative studies that have suggested the clinical efficacy of BCG in the treatment of human tumors and those that have measured the immunological consequences of BCG in man.

The three neoplastic diseases that have received the most attention from immunotherapists have been AML, malignant melanoma, and, most recently, lung cancer. After induction of a remission by intensive chemotherapy, Crowther and colleagues (*165*) administered BCG alone or with allogeneic tumor cells as maintenance therapy. These groups compared favorably with one given only chemotherapy, although none of the regimens significantly prolonged survival. In subsequent trials by the same group (*166*), an advantage was demonstrated for a regimen of chemoimmunotherapy, combining cytotoxic agents and BCG by vaccination plus irradiated allogeneic leukemia cells over a regimen involving chemotherapy alone. Both the duration of remission and survival were prolonged by the addition of immunotherapy to chemotherapy, in part because subsequent remission was more easily induced in patients given regimens including immunotherapy than in those maintained on chemotherapy alone (*167*). Although BCG was used alone successfully as maintenance therapy (*168*), the lack of concurrent controls in this study hinders definite conclusions about its efficacy. Vogler and Chan (*169*) intensively pretreated patients with BCG for 4 weeks before maintenance chemotherapy with MTX and found a significantly longer duration of remission in that group, compared with one given chemotherapy. Of the many studies with immunotherapy involving BCG with or without irradiated leukemia cells, few relied exclusively on immunotherapy for maintenance. Freeman et al. (*167*) detected no difference in the duration of the first remission but increased survival in the 8 patients given immunotherapy with BCG and leukemia cells, mainly because of a high rate of reinduction of remission.

The same benefit of immunotherapy has not been reproducibly demonstrated in ALL of children (*170–172*), despite Mathé's original encouraging report (*173*). Perhaps because chemotherapy alone is so effective in this disease, the additional benefit bestowed by BCG has been difficult for investigators to prove.

Intralesional injection of BCG has been effective in ameliorating tumor nodules in approximately 60% of the patients with melanoma [*see* table 1 in Bast et al. (*163*)]. Whether this is truly an augmentation of tumor-specific

immunity is debatable, inasmuch as the intense local inflammatory response accompanying rejection of the tumor is usually limited to the injected nodule or closely situated neighboring lesions. Distant visceral metastases, if present, have rarely been affected, and if absent, have developed despite regression of injected nodules. Thus systemic immunity may be irrelevant in the response to injected nodules, just as in the guinea pig hepatoma model, with direct local activation of macrophages and perhaps T-cells causing rejection. Nonspecific augmentation of systemic immunity to melanoma by vaccination with BCG, after surgical removal of a primary melanoma and tumor-containing regional lymph nodes, has been attempted by several groups. Gutterman et al. (*174*) found that using BCG with scarification was effective in prolonging the disease-free period in those who had a primary tumor of the trunk or extremities, but not of the head and neck. Historical controls rather than a concomitant, randomly assigned control group were used for comparison. The ostensible superiority of the BCG regimen in prolonging remission may have been due to its intralymphatic spread and consequent local antitumor effects rather than systemic augmentation of immunity, inasmuch as no BCG was applied in the head and neck region. Although the period of follow-up was only 2 years, and strict randomization was not performed, the study of Eilber and co-workers (*175*) is nonetheless encouraging. Whereas 42 control patients had a median duration of remission of 8 months, 84 subjects given BCG by multiprong vaccination for as long as 2 years had a significantly longer disease-free interval, with a projected median exceeding 42 months. Prospective randomized trials with BCG alone or combined with irradiated allogeneic cells are in progress and should help define the true efficacy of BCG. Thus far, it is apparent that the cure of microscopic metastases in melanoma by vaccination with BCG has been an elusive goal but significant advances have been made.

One of the most encouraging recent reports on immunotherapy with BCG has been that of McKneally et al. (*176*), who randomized 60 patients with bronchogenic carcinoma of the lung, stages I–III. Those patients with stage I (localized) cancer, each of whom received a single postoperative intrapleural injection of 10^7 viable U of Tice BCG, had a significantly lower rate of metastasis than those not receiving BCG. None of the 17 had a recurrence of disease versus 9 of 22 controls, 5 of whom died. Unfortunately, patients with stage II or III cancer were not benefited by intrapleural BCG. All BCG-treated patients also received INH to control spread of the mycobacteria. Such studies as these deserve wider randomized trials for confirmation. They represent a significant use of regionally administered BCG for its antitumor effects, primarily against tumor in regional lymph nodes and perhaps also against microscopic visceral metastases.

As an extension of the concept that immunotherapy is effective principally when the load of residual tumor is small, the basis for the use of BCG after primary surgical resection, some have advocated treatment with immunotherapy after reduction of systemic disease by chemotherapy (*177, 178*). Another rationale for this type of combined modality therapy is the possible protection of immunosuppression, myelosuppression, and suppression of IF production afforded by BCG. Whatever the theoretical basis, suggestions have been made that an increased duration of remission, though not an increased rate, have been effected by the addition of BCG to chemotherapy for melanoma and colorectal and metastatic breast cancer (*177*).

Immunological Effects of BCG in Man

Although the clinical efficacy of BCG has been suggested in several studies, it is noteworthy that the immunological correlates of a beneficial response generally have been lacking. Most studies that have measured immunity in patients treated with BCG have assessed nonspecific immunocompetence with microbial antigens to which the patients were presumed to be sensitized, such as *Candida,* mumps, *Trichophyton,* and PPD. Fewer studies have used the ability of a patient to be sensitized to a new skin test antigen, such as DNCB, as a measurement of increased immunocompetence. Chess and associates (*179*) found an increase in the magnitude of established DTH reactions after patients had been treated with BCG, but Mavligit and co-workers (*180*) detected no such effect. Furthermore, the latter group, studying 103 melanoma patients without gross evidence of disease, observed no change in the number of patients who were able to be sensitized to DNCB, nor in the intensity of reaction to the chemical. Lui et al. (*181*) saw no correlation between the effectiveness of BCG as immunotherapy and its effects on general immunocompetence, which was generally unaltered.

Lieberman and co-workers (*32*) studied 6 patients with cutaneous melanoma treated with intralesional BCG, of whom 4 had a good regression of injected nodules. Three of the 4 responders had an increase in their in vitro blastogenic response to melanoma antigens, and all 4 had an increased response to the T-cell mitogen PHA. The 2 nonresponders showed no such increases. Leukocyte migration inhibition in the presence of melanoma antigens was absent before therapy but was present in 2 of the 4 responders afterward. Rosette-forming cells were also increased in the responders, but only in 1 of the 2 nonresponders. These results suggest that intralesional BCG per se did not routinely cause an increase in specific and nonspecific parameters of CMI but that it did so primarily in responders to therapy, which perhaps indicated that the improved disease status was contributory to the improved immunological response.

Tumor-specific immunity was measured by several investigators, especially in patients receiving intralesional BCG. Generally, those patients with a favorable response to BCG have had lymphocyte-mediated cytotoxicity to melanoma cells, but elevation of that response was not always achieved by BCG treatment even in responding patients (*182–185*). Shibata et al. (*186*) discovered that oral BCG given to patients with melanoma increased CMI to allogeneic targets in 4 of 7 but increased antibodies in only 1 of 14. We have studied the effect of BCG on the reactivity of a patient to autochthonous or allogeneic melanoma cells, i.e., tumor-specific CMI. Mononuclear cells from the peripheral blood were tested in 7 patients given 1×10^6–

2×10^7 viable U Tice strain BCG intradermally or intralesionally (*47*). The CMI was not increased in the patients receiving intralesional BCG, despite the disappearance of the injected lesions in 2 of the 4 patients, but was increased by a mean of 20% above base line in the intradermally inoculated group, with a return to base line despite continued therapy. In a larger study (Mitchell MS, Merrill J, Mokyr MB: Unpublished data), 25 melanoma patients given Tice BCG by multiprong inoculations for 18 months showed poor correlations between their clinical responses, i.e., prolongation of the disease-free interval and the augmentation of CMI, which were inconsistent. Only 6 of 25 had a sustained elevation of CMI greater than 20% above base line, 13 had no significant change, 3 had a transient decrease in CMI with subsequent recovery, and the remaining 3 had a sustained decrease that was below their pretreatment level. Despite these in vitro findings, the same group has had improved median survival (33 mo) compared with historical controls that were cited above, and most of the group are still disease-free 2–3 years after the study. It is entirely possible that measurement of the reactivity of regional lymph nodes is a more accurate reflection of the effectiveness of BCG given into the skin, but obviously these nodes are inaccessible for serial studies of immunity. Notwithstanding the important and unresolved question of whether the cell-mediated cytotoxicity assay is a valid measurement of tumor-specific immunity, our results with the technique thus far have failed to provide evidence that BCG given into the skin of melanoma patients is capable of improving their CMI to homologous tumor cells.

Although some have claimed that improved survival has been produced by BCG in hematopoietic neoplasms, it remains uncertain whether improvement in the clinical response is immunologically mediated. One of the few groups (*187*) who attempted to measure tumor-specific responses in leukemia, which are difficult to demonstrate, claimed that 7 of 8 patients with CML who developed circulating antibodies to leukemia cells after BCG plus allogeneic irradiated myeloblasts had stability of their disease. In this setting, with antigen, BCG was designed for use as a true adjuvant, rather than as a nonspecific stimulator of the RES. However, proof that BCG augmented immunity is lacking, inasmuch as no controls were given myeloblasts alone for comparison.

Hazards and Toxicity

Local reactivity to BCG depends on the number of injections and the route of administration. Intradermal inoculations produce ulcerations locally, occasionally with regional lymphadenopathy, which may take several months to heal completely. Vaccination with a multiprong apparatus (Heaf gun) leads to slightly oozing lesions that are pruritic but usually are easily tolerable. Malaise lasting 24 hours, sometimes with a low-grade fever, is the most usual accompaniment of multiprong (Heaf gun) vaccination and is less severe than with intradermal administration (*188*). No more than approximately 2×10^7 BCG viable organisms are tolerable intradermally because of the severity of the local inflammation and ulceration, in contrast to 5×10^8 viable organisms applied to the skin by multiprong vaccination. However, the number of organisms actually entering the skin after the multiprong technique may be less than 2×10^7. Intralesional inoculation of BCG probably is tantamount to injection into the circulation because tumor nodules are highly vascular. Thus the number and severity of the complications by this route are greater. Fever in the range of 40°, chills, severe malaise, and anaphylactic reactions have been seen after intratumor injection (*189*). Granulomatous hepatitis has been documented by biopsy in approximately 12% of the patients receiving intralesional BCG (*190*), a process which appears to be allergic in nature, inasmuch as viable organisms have not been recovered from the lesions. At least 2 patients have died from massive Shwartzman-like reactions after repeated intralesional injections (*191*). Because BCG sensitizes mice to the effects of bacterial endotoxin (*192*), we presume it does so in man. It is these reactions, more severe in the tuberculin-positive or repeatedly BCG-vaccinated individual, that have stimulated the search for less toxic fractions that retain the beneficial effects of the whole organism. Enhancement of tumor growth by intradermally administered BCG has been suggested but not proved in man (*44*).

Conclusions and Projections

The specific properties of BCG, a useful immunological modulator, are only now being elucidated well after its value has been established empirically. Regardless of its future role as an adjunct in the treatment of cancer or other diseases, BCG can serve as an important prototype of a microbial modulator. Its activities in lymphoid and myeloid cells may be important indicators of which properties should be sought when screening similar agents for adjuvanticity or suppressive effects. From the clinical point of view, the further development of fractions of BCG that mimic its effects but lack its toxicity would be most desirable.

Modulation of immunity by BCG is the vectorial sum of its many effects on influx, trapping, and activation of lymphocytes and macrophages. Most of its overall effects have augmented immunity, but when suppressive activities are specifically measured, it is clear that they often coexist with adjuvant effects. It is likely, though perhaps a little disheartening, that carefully controlled "scientific empiricism" will be required if we are to determine the appropriate dosage schedules to achieve the desired effects because no single schedule is apparent that invariably causes adjuvanticity. For this reason, measurement of the immune response to the specific antigen in question, e.g., tumor cells, is mandatory in clinical immunotherapy trials to provide clinicians with a firmer base on which to judge the success or failure of the regimen.

Finally, the use of BCG as an adjuvant to specific antigen at appropriate schedules will almost surely be examined in humans when purified tumor antigens are available. This exploitation of the potential of BCG for increasing a specific immune response rather than simply its capacity as a chronic inflammatory stimulus locally or a

nonspecific stimulant of systemic immunity may prove to be a most important means of enabling the host to attack his tumor systemically and thus more completely.

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LEVAMISOLE

Introduction

Tetramisole, the racemic mixture of 2,3,5,6-tetrahydro-6-phenylimidazo(2,1-*b*)thiazole was introduced as a potent broad spectrum anthelmintic in 1966 (*1*). The anthelmintic spectrum of tetramisole is limited to various species of nematodes; there is no activity against cestodes or trematodes. Tetramisole and, more recently, the levoisomer levamisole (fig. 5-2) have been extensively and safely used in veterinary and human medicine (*2*). The demonstration by Renoux and Renoux (*3*) that tetramisole augmented the protective effect of a *Brucella* vaccine in mice triggered extensive investigation of the immunomodulating effects of tetramisole and levamisole. The results of clinical trials with levamisole have been summarized by tumor type in another section of the clinical report. Our purpose in this review is to provide background data on the pharmacology, metabolism, mechanism of action, immunologic profile, animal antitumor activity, and toxicity. The literature is voluminous (over 500 papers) but has been extensively reviewed (*4–7*). This document will be but a brief synopsis of these exhaustive reviews.

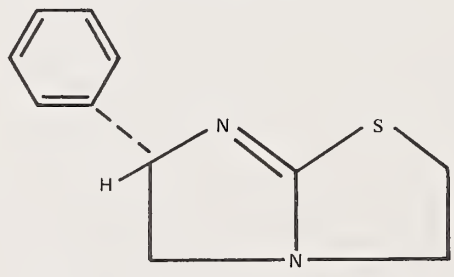

FIGURE 5-2.—Structure of levamisole.

Pharmacology

Levamisole (mol wt, 240.75) is a stable white crystalline powder; the hydrochloride salt is soluble in most organic solvents (*8*). In water, it is freely soluble and can hydrolyze, and the rate of hydrolysis increases with increasing pH or temperature. At a pH of 4 or less, 3–18% aqueous solutions of levamisole have remained stable for more than 40 months. Levamisole precipitates at pH 7.5.

Metabolism

Animal

Levamisole is well absorbed in several species by the oral, sc, or im routes (*9*). Plasma half-life of the unchanged drug ranges from 1 to 4 hours. The drug is widely distributed and can be detected in all tissues and fluids, with the highest levels in the liver and kidney. These are the organs involved in levamisole metabolism. Levamisole is extensively metabolized and rapidly removed from the body. In rats, less than 1% of the administered radioactive dose remains in the body after 8 days. Species or sex differences in the kinetics of the drug are not obvious. In rats, 46% of the radioactive dose is excreted in the urine and 40% in the feces within 48 hours. The 8 metabolites that have been identified are less toxic than levamisole and have no potent anthelmintic effects.

Human

A single oral dose of 150 mg of [^{3}H]levamisole produces a peak plasma level of 0.49 ± 0.05 μg levamisole/ml at 2 hours. This is the same concentration as is required in vitro for restoration of depressed phagocytic and T-lymphocyte function to normal. At the peak level, the radioactivity due to unchanged drug is only one-third of the total plasma radioactivity. The plasma half-life of levamisole is 4 hours. The peak plasma levels differ little between individuals, which indicates that the drug is well absorbed.

Approximately 60% of the administered dose is excreted in the urine by 24 hours (70% by 72 hours). In the same period, only 4% of the administered radioactivity is excreted through the feces. Extensive metabolism is indicated by the fact that only 6% of the urine radioactivity and 4% of the fecal radioactivity was due to unchanged levamisole.

Mechanisms of Action

The anthelmintic property of levamisole results from its ability to stimulate autonomic ganglia. It also increases heart rate and strength of contraction (*5, 8*). Levamisole has no direct effect on bacteria, viruses, or fungi. In vitro it is not cytotoxic to normal or neoplastic cells.

Levamisole contains an imidazole ring, and it may function like imidazole in affecting enzymes controlling cyclic nucleotide levels in lymphocytes to affect their function and induce immunoregulation (*10–12*). Thus imidazole and levamisole, which are not themselves mitogenic, elevate cGMP levels in lymphocytes in vitro and enhance their proliferative response to mitogens or foreign cells.

Immunological Profile of Levamisole In Vitro and In Vivo

This area of research has been extensively reviewed by Symoens and Rosenthal (*5*). Their conclusions are as follows:

In Vitro Test Systems

Phagocytosis.—Levamisole increased phagocytosis by PMN cells or macrophages when added to these cells or given to donor animals and humans. The effect was most pronounced on hypofunctional cells from patients and weak or absent on cells from normal donors.

Chemotaxis.—Chemotactic responsiveness of PMN cells and monocytes from patients with defective leukocyte motility could be enhanced by levamisole added in vitro or given in vivo.

Migration inhibition and lymphokine production.—Leukocyte migration inhibition in response to antigenic stimulation could be restored when levamisole was administered to anergic patients or added to their cells in vitro. The effect of the drug was most pronounced when the antigen used was present in suboptimal concentrations. Little or no effect was observed in healthy donors with normal skin reactivity or with levamisole alone without antigen. The drug seemed to augment the production of soluble mediators of delayed hypersensitivity in mitogen-stimulated human peripheral lymphocytes in vitro. The drug did not induce such a production in the absence of antigen.

Lymphocyte stimulation.—Levamisole increased nucleic acid or protein synthesis in resting lymphocytes as well as in mitogen, antigen, or allogeneic cell-stimulated T-lymphocytes. The B-cell response did not appear to be affected by this agent.

The effects of the drug were most pronounced on hypofunctional cells from old, diseased, or immunosuppressed donors. Following irradiation in man or cytotoxic treatment in mice, levamisole applied at a critical time restored depressed lymphocyte responsiveness. Too early administration of levamisole after cytotoxic treatment induced further depression and delayed restoration of the lymphocyte response.

Peripheral, splenic, and T-lymphocytes from adult humans or animals seemed to respond to levamisole in vitro and in vivo under appropriate conditions, although various investigators present contradictory views in this matter. After in vivo restoration of lymphocyte responsiveness by levamisole, no further in vitro stimulation could be achieved. Spleen cells from nude mice and spleen cells from mice pretreated with anti-thymocyte serum showed no or poor response to levamisole in vitro. Splenic T-cells from thymectomized mice responded well.

Lymphocyte suppressor activity.—Human splenocytes and thymocytes incubated with mitogen in the presence of levamisole suppressed the MLR to a significantly higher degree than cells not exposed to the drug. In contrast, the inhibition of the response of lymphocytes to PHA, which is induced by supernatant fluids of mononuclear cells from anergic patients, was abolished or decreased by levamisole in vitro.

Lymphocyte or macrophage cytotoxicity.—Lymphocyte cytotoxicity against allogeneic cells was markedly enhanced in the presence of levamisole. The drug effect, either before or during sensitization, resulted in more target allogeneic cells killed per lymphocyte.

In patients with melanoma, levamisole increased the preexisting specific antitumor lymphocyte cytotoxicity, prolonged lymphocyte cytotoxicity induced by a tumor cell vaccine, and even induced this cytotoxicity in response to suboptimal and normally ineffective doses of the vaccine.

However, such an effect could not be obtained in levamisole-treated mice sensitized with allogeneic cells, or syngeneic or allogeneic tumor cells. In the latter type, cytotoxicity was even decreased. Levamisole failed to restore lymphocyte cytotoxicity in virus-immunosuppressed mice and failed to increase macrophage cytotoxicity in normal mice.

Lymphocyte counts.—Levamisole restored to normal the number of T-cells as determined by the estimation of E-rosette-forming cells in patients in whom these numbers were reduced. When a reduction of T-cells was accompanied by increased numbers of Ig-bearing cells, null cells, or EAC-rosette-forming cells, a drop in the numbers of B-cells or null cells was observed following levamisole treatment. The increase in T-cells and reduction in B-cells or null cells occurred without any significant change in the absolute lymphocyte counts. Furthermore, levamisole had no effect on the number of murine spleen and human fetal E-rosette-forming cells or Ig-bearing cells in vitro but increased the number of E-rosette-forming spleen cells of thymectomized mice in vivo.

Total T- and B-cell counts that were already within normal range did not change significantly under levamisole therapy, except at extremely high dose levels. When, after treatment, E-rosette formation was restored to normal, no further in vitro stimulation could be achieved. Preliminary observations suggest that, in normals, levamisole significantly increases the number of active T-cells in peripheral blood as estimated by the modified E-rosette assay of Wybran.

The drug seemed to remove a blocking substance (probably apoferritin) from the surface of lymphocytes of patients with Hodgkin's disease. The rosettes formed by human PBL in the presence of levamisole disintegrated more slowly than in untreated controls.

Jerne plaque test.—The number of PFC in the direct (19S) as well as in the indirect (7S) assay was often increased when levamisole was present in the cultures or added to the sensitized donor animals. By itself, however, the drug failed to increase background numbers of PFC. Yet it could partially restore the depression of PFC formation induced by cytotoxic agents.

Immunoglobulin levels and antibody formation.—In the vast majority of the animal and human models tested, levamisole had little or no effect on existing serum Ig levels or on specific antibody production to the particular bacterial, viral, or cellular immunogen.

Immune complexes.—A fall in circulating immune complexes was observed in rheumatoid arthritis patients after treatment with this agent.

Complement and acute-phase proteins.—In patients in whom the total hemolytic complement was increased, the

administration of levamisole resulted in a fall of the values to normal range. A transient rise in complement levels was observed early in the treatment of elderly people and of patients with various diseases, but no change in complement values was found in treated healthy people under the age of 50 years.

Levamisole decreased C-reactive protein levels in patients with rheumatoid arthritis (358) and increased them in cancer patients; also, it increased haptoglobin levels in the healthy volunteers.

Interferon.—Levamisole induced the production of IF by normal HLC. In contrast, no antiviral effect indicative of IF induction was detected in levamisole-treated mice or in fibroblast cultures to which the compound was added.

Cyclic nucleotides.—An increase in cGMP and a decrease in cAMP in mice spleen T-cells were observed in vitro in the presence of levamisole. Similar effects could be seen in lymphocytes and PMN cells from patients.

In Vivo Test Systems

Delayed hypersensitivity.—Skin tests for DTH to a variety of antigens were frequently restored by levamisole in anergic patients and boosted in subjects whose skin reactivity to antigenic stimulants was reduced. Such observations were made in healthy elderly subjects and patients with cancer, leprosy, or various other nonmalignant diseases.

The drug prevented a suppression of delayed skin hypersensitivity to tuberculin commonly seen after influenza vaccination in elderly people, and it reversed the cortisone-induced inhibition of the DTH in rabbits.

Blood clearance in colloidal particles.—The blood clearance of colloidal particles was enhanced by levamisole when the RES was deficient as observed in humans who are ill, in aged or cortisone-treated animals, and in certain strains of animals. Levamisole appeared to restore RES function rather than to stimulate it above normal functional levels of activity.

Skin graft rejection.—No alteration of allogeneic or isogeneic skin graft rejection time was noticed in mice, except at high doses of levamisole, when rejection time could be shortened.

Graft-versus-host reaction.—This reaction to splenic cells was increased in mice when the donor of the recipient had been treated with levamisole.

Adjuvant disease of the rat.—Levamisole did not influence the primary inflammatory response to the immunization of rats with complete Freund's adjuvant. However, it significantly increased the systemic disease and arthritis induced by this agent.

Experimental allergic encephalomyelitis.—The incidence as well as the severity of this disease in rats was markedly increased by levamisole. In animals treated with the drug alone, i.e., without complete Freund's adjuvant, only mild histologic changes typical for this disease were seen.

NZB/NZW nephritis.—NZB/NZW mice treated with levamisole lived significantly longer than did control mice and proteinuria and antinuclear antibodies developed more slowly.

Animal Antitumor Effects

Levamisole does not appear to influence the primary growth and dissemination of the tumors (*5*).

Amery (*6*) reviewed the animal tumor data regarding the use of levamisole in conjunction with cytoreduction therapy. Mice with systemic LSTRA leukemia treated with BCNU had a twofold increase in survival time and a 30% survival rate. Levamisole alone was therapeutically ineffective because it produced no survivors, but when administered 3 and 6 days after BCNU treatment, a significant number (90% and 70%, respectively) survived. Delaying treatment with levamisole for 10 days at the 5-mg/kg dose was ineffective. Therapeutic failure could be attributable to the fact that animals treated with BCNU had begun to relapse. However, the therapeutic efficacy of delayed treatment was restored by an increase in the dose of levamisole. In a second tumor model, the Lewis lung adenocarcinoma, the effect of Me-CCNU plus levamisole was tested. Three parameters were elevated in this study: effect on life-span, effect on primary tumor weight, and the number of metastatic lung lesions that occurred. Levamisole alone was ineffective in increasing life-span; however, decreases in tumor weight and number of lung lesions were noted. The combined treatment of Me-CCNU and levamisole proved more effective than Me-CCNU alone, which resulted in a signifcant increase in life-span (30 to 55 days) and decreases in primary tumor weight (6.6 to 5.3 mg), and the number of lung lesions (18 to 14).

A summary of animal tumor models with cytoreductive therapy followed by levamisole is presented in table 5-1. Based on the results of these studies, levamisole seems to be most effective when used as an adjuvant after cytoreductive therapy.

The dose effect of levamisole was also analyzed, i.e., a determination of whether the dose of levamisole, when used singly or combined with cytoreductive therapy, was important. The analyses shown in tables 5-2–5 are based on various mouse tumor models because only fragmentary data were available for other species. Table 5-2 shows the results of a comparison between levamisole alone and as an adjuvant to chemotherapy. When the former regimen is used the 3- to 5-mg/kg dose appears to be critical: A reasonable number of positive responses were attained (9 or 21 compared with 1 of 11 with other doses). More positive responses were achieved at all doses tested when levamisole was combined with chemotherapy than when it was used alone. Treatment with it as an adjuvant appears superior to that with the drug alone.

Another analysis was based on the medical survival time of animals with slowly and rapidly growing tumors that received therapy with levamisole alone or as an adjuvant to cytoreductive therapy (table 5-3).

Positive results were obtained in 77 of 382 experiments with rapidly growing tumors and 59 of 200 with slowly growing ones. The greater effect on the latter tumors represents a significant difference. In the overall assessment of levamisole dosage, a range of 3 to 10 mg/kg appears favorable for both types. The evaluation of results reported for the effect of levamisole alone on primary tumor growth and metastasis formation indicates that the primary tumor

TABLE 5-1.—*Secondary growth and dissemination of tumors*

Tumor model	Animal species	Cytoreductive therapy[a]	Effect[b]
L1210 leukemia	Mice	Me-CCNU	+
Moloney LSTRA lymphoid leukemia	"	BCNU, CPP	+
Moloney MCAS-10 lymphoid leukemia	"	BCNU	+
Graffi L leukemia	"	BCNU, CPP	+
Lewis lung carcinoma	"	Me-CCNU	+
MCA-induced fibrosarcoma	"	BCNU, CPP	+
KHT fibrosarcoma	"	Radiotherapy	+
Meth A tumor	"	CPP	+
Spontaneous mammary tumor KSP-1	"	Me-CCNU	+
EMT-6 mammary tumor	"	Radiotherapy	+
Melanoma B16	"	Surgery	+
MC-1 sarcoma	Rat	CPP	+
Fortner's melanotic melanoma 1	Hamster	Surgery	+
L1210 leukemia	Mice	Various	0
Lewis lung carcinoma	"	Surgery	0
MO_4 sarcoma	"	CPP	0
6C3HED lympho-sarcoma	"	Radiotherapy	0
L 311 lymphoid leukemia	Rat	Adriamycin	0
ISIS 208 and ISIS 130 leukosarcoma	"	CPP	0
Epithelioma	"	Surgery	0

[a] Various = CCP, BCNU, 5-FU, MTX, or adriamycin.
[b] + = effect; 0 = no effect.

was influenced only marginally, as shown in 14 of 78 tests (table 5-4). Because few studies were conducted at the 1- to 3- and 5- to 10-mg/kg doses of levamisole, no conclusions concerning a dose–response relationship can be drawn.

Side Effects

Nonhematological

Pinals (*13*) reviewed the nonhematological adverse reactions in 3,900 patients (989, rheumatic diseases; 601, other inflammatory diseases; 888, infectious diseases; 1,179, cancer; 243, other diseases) treated with levamisole. These are summarized in table 5-5.

TABLE 5-2.—*Levamisole alone versus levamisole adjuvant to cytoreductive therapy*

Levamisole dose, mg/kg	Positive responses			
	Levamisole alone		Levamisole plus chemotherapy	
	No.	Percent	No.	Percent
1–3	0/2	0	1/4	25
3–5	9/21	42	21/34	61
5–10	1/8	12	5/11	45
10	0/1	0	3/3	100

TABLE 5-3.—*Effect of levamisole on transplantable tumors with different growth rates in mice*[a]

Levamisole dose, mg/kg	Positive responses					
	Slowly growing		Rapidly growing		Both	
	No.	Percent	No.	Percent	No.	Percent
1	13/59	22	1/35	2	14/94	14
1–3	13/27	48	8/97	8	21/124	16
3–5	20/63	31	39/161	24	59/224	26
5–10	7/17	41	25/80	31	32/97	33
10	6/34	17	4/9	40	10/43	23

[a] The median survival time for the slowly and rapidly growing tumors was 20 days.

The nonhematological side effects include 1) sensorineural reactions, the most common of which is alterations in taste and smell; 2) idiosyncratic or allergic reactions, such as a rash and a febrile influenza-like illness, and 3) gastrointestinal symptoms. The sensorineural reactions are seldom of sufficient severity to require discontinuation of treatment. Rash and febrile illness have resulted in withdrawal of levamisole in 7 and 1.5% of the patients, respectively, but they usually remit spontaneously within a brief interval and are not hazardous except when associated with agranulocytosis. Rash due to levamisole occurs more frequently in patients with rheumatic diseases than in other conditions. Gastrointestinal symptoms are mild, quickly reversible, and drug related. Some controlled trials have shown a similar frequency of gastrointestinal symptoms in the placebo group.

Hematological

When first used as an anthelmintic, levamisole caused no serious hematological side effects (*14*). The first reports of agranulocytosis concerned patients with rheumatoid arthritis who had been treated daily for several months (*15*). Soon thereafter, 3 fatalities were reported (*16, 17*). In 71 reports in which 1,131 rheumatic disease patients received levamisole, 989 patients were screened for side effects and these were noted in 4.9%. The frequency of hematological side effects seems much higher in rheumatoid arthritis than in other diseases in which the incidence varies between 0.2% in patients with infectious disease and 2% in those with cancer.

Mielants and Veys (*14*) collected data (by questionnaires) on 88 patients with agranulocytosis (selective fall of

TABLE 5-4.—*Effect of levamisole on metastasizing transplantable tumors in mice*

Levamisole dose, mg/kg	Positive responses			
	Primary tumor		Metastasis formation	
	No.	Percent	No.	Percent
1	6/32	18	7/32	21
1–3	1/7	14	4/7	57
3–5	5/34	14	16/34	47
5–10	2/5	40	3/5	60

TABLE 5-5.—*Percent adverse reactions to levamisole*[a]

Type of reaction[b]	Rheumatic diseases		Other diseases	
	Reaction	Withdrawn	Reaction	Withdrawn
Sensorineural				
Sensory stimulation	6.2	0.5	4.1	0
Hyperalert state	1.8	0	0.9	0
Insomnia	1.4	0	1.2	0
Headache	1.3	0.2	2.0	0
Dizziness	2.9	0	1.1	0
Idiosyncratic				
Rash	14.8	7.0	2.0	0.5
Febrile illness	3.9	1.5	3.1	1.0
Stomatitis	1.9	0.3	0.2	0
Gastrointestinal				
Nausea	5.0	1.3	8.3	0.4
Intolerance	5.7	0.8	4.2	0.4
Anorexia	2.7	0	1.2	0
Vomiting	2.1	0.2	3.8	0
Diarrhea	0.3	0.1	1.4	0

[a] No. of patients = 989 with rheumatic diseases and 2,911 with other diseases.

[b] Rare reactions include tremor, vertigo, visual hallucinations, somnolence, confusion, depression, lymphadenopathy, lupus-like reaction, allergic vasculitis, sicca syndrome, increased transaminase levels, azotemia, proteinuria, polyuria, and angioneurotic edema.

neutrophils below 20%), 43 patients with leukopenia (leukocytes below 3,000/mm^3 but with more than 20% neutrophils), and 3 patients with thrombocytopenia (platelets less than 50,000/mm^3). Agranulocytosis could not be related to dose or schedule of levamisole, concomitant medications, or to the age or sex of the patients. A significant genetic predisposition related to HLA-B27 was noted: B27 was present in 8 of 16 patients with agranulocytosis in whom the HLA pattern was studied. This is significantly more than the expected frequency. Patients with rheumatoid arthritis who developed agranulocytosis after treatment with levamisole usually showed an excellent therapeutic response to the drug. Agranulocytosis was always spontaneously reversible when treatment was discontinued. Eight fatalities were reported, however, secondary to intercurrent infection.

The 43 patients who experienced leukopenia differed in that no increased prevalence of HLA-B27 was observed, bone marrow showed no changes, and agranulocytosis did not develop with continued treatment.

Summary

Levamisole is the first member of a new series of chemically defined agents with stimulating activity on the immune system, preferentially of the cell-mediated type. The exact mode of action remains unknown. Levamisole has been therapeutically effective in various animal models when used following cytoreduction therapy. The vast clinical experience has been summarized in another section of this report. Side effects are modest and, except for infrequent reversible agranulocytosis, are not clinically significant.

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MISMATCHED BASE-PAIR POLYNUCLEOTIDE

Considerable data now indicate that the administration of IF in vivo in man can result in tumor regression or

prolongation of remission and survival in patients with cancer. Clinical trials with IF have been initiated after investigators demonstrated that IF 1) could inhibit the proliferation of normal and malignant cells in vitro, 2) did inhibit the proliferation of human tumor cells in vitro, and 3) could slow the rate of tumor development and growth in various animal tumor models (*1*). Because IF is a natural host defense substance, produced in response to viral infection as well as to other noxious stimuli, its antiviral and antitumor activities may be related in several important ways. Both animal and human experience indicate it is nontoxic, which provides a strong rationale for its use in therapeutic trials in man. After the pioneering effort of Strander and co-workers (*2*), an increasing number of investigators reported biological and therapeutic activity for IF in human cancer (*3*). Results clearly indicate the need for extensive research on its therapeutic use in man and also for the development of safe, nontoxic, and potent IF inducers. The combined use of IF and an inducer may result in augmented antitumor activity.

Patients with osteogenic sarcoma were treated with leukocyte IF after surgery and compared with concurrent and historical controls (Brostrom LA: Personal communication). The estimated 5-year survival in this adjuvant trial is 50% in the treated patients compared with 24 and 17% in the control groups. Three patients with pulmonary metastases of osteogenic sarcoma were given leukocyte IF and partial remissions were observed in 2 (Kishida T: Personal communication).

Of 7 patients with acute leukemia who received leukocyte IF daily for several weeks (Hill NO: Personal communication), all showed clearing of peripheral blood leukemia cells, and 2 with ALL entered complete remission. Strander (personal communication) administered leukocyte IF to 7 patients with laryngeal papillomata and all 7 showed at least partial if not complete remissions. Several other workers studied leukocyte IF therapy of multiple myeloma. Strander also treated 12 patients who had not received prior chemotherapy and induced 2 complete and 5 partial remissions (personal communication). Other clinicians (Gutterman JU: Personal communication) reported that 5 partial remissions were induced in 9 patients.

Merigan (*4*) treated 3 histocytic lymphoma patients who did not respond and 3 patients with nodular, poorly differentiated lymphoma who had partial remissions. Gutterman (submitted for publication) treated 8 patients with various lymphomas and noted 6 partial remissions, and 17 patients with stage IVB breast cancer. Seven patients who entered partial remission were mainly those who had responded previously to chemotherapy and had limited tumor burdens. Several clinicians treated cutaneous tumor nodules including melanoma and cancers of the breast and prostate with intralesional injections (Hatif D; Horoszewicz JS: Personal communications). In almost every patient, the injected nodule regressed, and biopsies showed mononuclear cell infiltration. Immunological studies of some patients who received systemic leukocyte IF have shown increased NK cell activity (Einhorn S: Personal communication). These data clearly indicate the potential for a safe, effective IF inducer in cancer therapy.

With the past experience of investigators with IF inducer therapy, it is important that we point out that all the active nonspecific immunotherapeutic agents are IF inducers to some extent (*5*). Schultz and co-workers (*5*) have proposed that the clinical activity and immunological effects of the inducers, such as increasing macrophage function, NK cell activity, ADCC activity, etc., might be related to the IF.

Agents more commonly thought of as IF inducers such as pyran copolymer, poly I:C, and poly I:C-L-lysine have been used in clinical trials to a limited extent. Clinical trials have been based on extensive animal model work showing that these compounds are IF inducers, macrophage activators, activators of the RES, immunostimulators, and that they can cause regression of established animal tumors or can prolong remission and survival after conventional therapy in the models (*6*).

Most human or clinical studies of IF inducers revealed considerable toxicity with little evidence of antitumor activity. These failures have been related to the fact that 1) the agents are indeed toxic, 2) they were administered at times in excessive doses, 3) their administration was not guided by measurements of the level of IF they induced, and 4) the patients selected were at times far advanced with large tumor burdens and many preexisting complications. High doses of pyran copolymer were administered to over 30 patients daily for up to 4 weeks (Regelson W, Schnider BI, Colsky J, et al: Submitted for publication); significant toxicity, fever, and chills were noted. One patient achieved a partial remission. Robinson and co-workers (*7*) treated a series of advanced chemotherapy refractory cancer patients with poly I:C at 0.3–75.0 mg/m^2. Significant toxic side effects were noted including fever, chills, nausea, vomiting, headache, prostration, and cytopenia; there were no remissions (*7*). More recently, Levine and Levy (*8*) gave a series of patients poly ICLC (poly I:C stabilized with poly-L-lysine). This more stable, nuclease-resistant agent was hypothesized to be a more potent IF inducer in man. Unfortunately, severe toxicity was again noted for this analog (*8*); however, 1 patient with ALL entered complete remission.

A major effort in the development of IF inducers for antiviral or for antitumor therapy has been limited because the best inducer, theoretically, i.e., synthetic double-stranded RNA which can induce high levels of IF, has many serious toxic side effects in animal models, including acute lethality, pyrogenicity, suppression of hematopoiesis, coagulopathy, and direct cytotoxicity (*9*). These side effects observed in animal systems have also been seen clinically, as outlined above.

Ts'o and colleagues (*10*) and Carter et al. (*11, 12*) described a group of polynucleotide duplexes having mismatched base pairs ($rI_n \cdot R(C_{12}, U)_n$, $rI_n \cdot r(C_{29}G)_n$, and others) which are hydrolyzed five to nine times faster by serum nucleases than normal double-stranded RNA ($rI_n \cdot rC_n$). The mismatched poly I:C with the formula $rI_n \cdot r(C_{12}U)_n$ is prepared as follows: The single-stranded polymers are synthesized from the appropriate nucleotide diphosphates by incubation with polynucleotide phosphorylase. After deproteinization, the complementary strands of polyinosinic acid (6.35) and the copolymer $r(C_{12}U)_n$ (7.2S) are annealed by mixing 1:1 stoichiometric

amounts of the complementary strands in a solution of the duplex with a concentration of 1 m*M* total base at 4° C for 90 minutes. A more extensive description of the standardization and synthesis may be found in earlier references (*12*). These compounds had comparable IF induction and in vitro antiviral and in vivo antiviral activity (protection activity) in the mouse as poly I:C but had markedly reduced lethality, pyrogenicity in the rabbit, mitogenicity, and splenomegaly- and cytopenia-inducing activity. The compounds have not been tested for their antitumor activity.

These results suggest that these compounds may be effective and nontoxic IF inducers in man. Furthermore, because IF inducers clearly have antitumor activity in animal models and IF has antitumor activity in human beings, these agents should be studied with highest priority. Inasmuch as poly I:C and other IF inducers have been administered safely to patients (albeit with considerable toxicity), the mismatched inducers should be as active but with markedly diminished toxicity. Investigators should perform phase I–II human studies of the mismatched poly I:C duplex for its toxicity, MTD, IF-inducing capacity, immunological effects, and antitumor activity.

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DIVINYL ETHER-MALEIC ANHYDRIDE (PYRAN) COPOLYMERS IN CANCER THERAPY

Divinyl ether and maleic anhydride form a 1:2 copolymer commonly known as pyran copolymer. Crude pyran copolymer is a straight chain, C-C backbone anionic polymer, which has an average mol wt of 30,000, a major mol wt range of 18,000–32,000, and a full spectrum of weights from 5,000 to 100,000 (*1*). Subfractions of pyran copolymer have been produced with narrow range mol wt of 12,500 to 15,500, 21,300, 32,000, and 52,600, respectively, referred to as MVE 1–5 (*2*). Crude pyran copolymer has a number of important biological activities including IF induction, macrophage activation, immunostimulation and immunosuppression, and antitumor activity; it also protects animals from subsequent infection (*1, 2*). For these reasons, it is considered to have significant potential for cancer treatment. Clinical studies of crude pyran copolymer have been limited because of its excessive toxicity. Attention is now focused on the MVE fractions because of their reduced toxicity; MVE-2 has been entered into clinical trials.

Extensive data from animal experiments indicate that pyran can protect animals from subsequent infection. Protection has been seen for bacterial infections with *Pasteurella (Francisella) tularensis* (*3*), *L. monocytogenes* (*4*), *S. aureus* (*5*), and *Klebsiella* (*6*) species. Protection against *Cryptococcus neoformans* (*7*), *Plasmodium* species (*8*), and *Toxoplasma infection* (*9*) has also been observed. Extensive studies have been done on protection against viral infections. Pretreatment with pyran will protect mice against infection with the following viruses: Friend leukemia (*10*), Moloney sarcoma (*11*), Rauscher (*12*), herpes simplex (*13*), Newcastle disease (*14*), and foot and mouth disease (*15*). With the latter virus, important species differences are noted, in that mice and guinea pigs are protected, whereas cattle and pigs are not (*16*). The protection is manifested as reduced lethality and prolonged survival compared with controls. In addition, viral oncogenesis is prevented or delayed in mice for Rauscher (*17*) and Friend (*18*) leukemia viruses and polyoma virus-induced tumors (*17*). The effect of pyran is partly T-cell independent, and protection has been observed in mice deprived of T-cells by thymectomy, radiation, and bone marrow reconstitution (*13, 19*). Pretreatment is mandatory; treatment after infection is established is not effective. Treatment effect is not the result of the antiviral activity of IF but probably related to macrophage activation (*20*). The effect is long lasting and has been observed to persist for weeks up to 2 months after a single dose of pyran. Apparently, this activity in itself would be worthy of investigation in cancer patients undergoing myelosuppressive chemotherapy.

Extensive data also exist on tumor growth inhibition by pyran. Some of the tumors, the growth of which has been inhibited by administration of pyran after tumor inocu-

lation, include Ehrlich ascites tumor (*21*), Rauscher virus-induced leukemia (*22*), Lewis lung carcinoma [(*23*); in which local growth and metastases are both reduced], melanoma B16 (*24*), various MCA-induced sarcomas and fibrosarcomas in the mouse (*25, 26*), spontaneous and first-generation transplanted mouse mammary carcinoma (*27*), and the Madison 109 tumor (*28*). Of the antitumor agents studied, pyran was the most effective in this latter tumor. In addition to tumor growth inhibition when administered alone, the combination of pyran copolymer with known anticancer drugs proved to be additive or synergistic in LSTRA leukemia (*29*), Lewis lung carcinoma (*30*), and spontaneous mammary carcinoma (*31*). Furthermore, pyran copolymer is additive or synergistic with radiotherapy in MCA-induced fibrosarcoma and mastocytoma carcinoma in the mouse (*32*). Both increased complete remission and increased cure rates have been observed. Finally, administration of pyran during the premalignant period retards the development of AKR leukemia (*33*), MCA-induced fibrosarcoma (*34*), and DMBA-induced squamous cell carcinoma in the oral cavity of the golden Syrian hamster (*35*).

The mechanism of action of these agents, studied in detail, is strongly associated with macrophage activity and increased macrophage infiltration of tumors as observed in pyran-treated, tumor-bearing animals (*36*). Generally, in comparisons of the activity of various anionic polymers and other active, nonspecific, immunotherapeutic agents, the degree of macrophage activation and antitumor activity correlate directly. Several studies have demonstrated that the mechanism of action is not directly related to IF induction, although subsequent and long-lasting macrophage and NK cell activation by IF may be important mechanisms (*37*). The activity of pyran copolymer is retained partially in T-cell-deprived animals, which suggests that T-cell-mediated cytotoxicity is not an important component of its activity (*38*). The antitumor activity is closely associated with molecular weight; the agent below 1,000 daltons is not active, but one of 10,000 daltons or greater is active (*39*). Above 10,000, toxicity increases but antitumor activity does not. The defined molecular weight-range polymer preparations retain their antitumor activity (*1*).

Tumor enhancement has also been observed with pyran copolymer. This includes prolonged leukemia allograft survival in mice (*40*), accelerated skin tumor induction in rats by pyran (*41*), and augmentation of Friend leukemia virus splenomegaly when the pyran is given iv (*42*). In summary, these data suggest that immunotherapy with defined MVE fractions of pyran should be investigated clinically.

Pyran has been observed to have a variety of host defense modifying effects. Thus it has adjuvant activity when administered with antigen for heightened antibody response to SRBC (*43*), tumor cells, e.g., L1210 leukemia, (*44*), and foot and mouth disease virus (*45*). Pyran has potent RES augmenting activity including increased phagocytosis, particle clearance [carbon Re glycolipid, RBC (*46*)], and removal of polymeric plutonium from the mouse liver (*47*). The response is biphasic because 2 days after a dose of pyran, RES activity is depressed followed by heightened activity compared with the base line. Pyran also has increased macrophage mobilization (*48*), and, most importantly, it is a potent and long-lasting macrophage activator. This includes activation for specific and selected tumor cell killing versus the killing of normal cells (*49*). Macrophage activation peaks at 5–7 days but can last weeks to months after a single dose (*50*). One can demonstrate the antitumor activity of pyran-activated macrophages by mixing tumor cells and activated macrophages and inoculating them in vivo after which delayed tumor growth is seen and also by injecting pyran intralesionally into tumor nodules which is associated with tumor nodule regression and macrophage infiltration (*51*). In contrast to the above activating results, pyran administration in vivo results in transient diminution of lymphocyte blastogenic responses to PHA and PWM in mice (*52*) and decreased cell-mediated cytotoxicity to allogeneic tumor cells (*53*). Of the interferon inducers (*1, 2*), this polymer is one of the more potent.

Distribution studies of [^{14}C]pyran show that it is taken up mainly by the RES in the liver and spleen (*54*); clearance studies demonstrate it can persist for long periods; and 25% remains after a single dose in vivo at 5 weeks.

The structural requirements for activity, which have been defined, include a chemically stable C—C backbone, a high mol wt ($\geq$10,000), and a regular dense sequence of negative charges on a long-chain backbone (*55*). The mechanisms of protection against infection have been associated with macrophage activation including increased phagocytosis (*56*), chemotactic reactivity probably related to complement activation (*57*), macrophage spreading (*58*), and ADCC activity (*59*). This increased activation in turn may be related to IF induction (*60*). All the activities are related to molecular weight. At greater than 60,000 daltons, toxicity predominates, whereas at lower ones that approximate 15,000, antitumor activity is retained (*1, 2*). Furthermore, although the higher molecular weight pyran depresses RES activity, that with the lower ones stimulates it (*61*). Finally, pyran is a potent inhibitor of RNA-dependent DNA polymerase in certain tumor cells, which may account for some of its antitumor activity (*62*). The mechanisms of immune suppression by pyran is related to the induction of suppressor cells active against both T- and B-cell proliferative responses and are presumably macrophage suppressor cells (*63*).

Pyran has significant toxicity, including lethality to mice at high and low doses, weight loss, and hepatosplenomegaly. Animals that received a single dose are highly sensitized to the lethal effects of subsequently administered endotoxins (*1, 2*). Because it inhibits hepatic microsomal mixed function oxidases, pyran is associated with a marked slowing of barbiturate metabolism (*64*). It has a heparin-like anticoagulant effect (*1, 2*).

In one clinical trial, crude pyran copolymer was given to 4 patients (at 60 mg/kg) who had viral encephalitis (*65*); no therapeutic benefit was observed. It did induce IF but also caused reversible renal failure. In another study, this crude copolymer was given to 4 patients at 18 mg/kg and resulted in anticoagulation but no bleeding (*66*). The most extensive studies, conducted by Regelson and co-workers (*67*),

included a phase I study of 36 courses in 29 patients with solid tumors. In 23 of these, the dose was under 4 mg/kg, but in the others, it ranged up to 16 mg/kg. In daily treatment for up to 62 days (median 14 days), the drug was given iv in 1 hour. No severe toxicity was noted, but the patients all had fever, chills, and malaise, and, at doses over 8 mg/kg, most had thrombocytopenia. Regelson and associates then did a phase II study with 12 mg/kg in 33 patients with solid tumors. Treatment was daily for 1–29 days at a total dose of 350 to 33,000 mg, for a median total dose of 3,000 mg; median duration was 9 days; median survival from the start of therapy was 16 days. Most of these patients had fever, chills, and malaise; 10 of 33 had thrombocytopenia, 9 leukopenia, 3 hypertension, 3 acute CNS symptoms, including transient visual loss. One patient died of gastrointestinal bleeding. This is the only death that could be attributed to the agent. Inasmuch as 11 of the patients were far advanced (median survival, 16 days), these data are hard to interpret. Only 1 partial remission was attributed to therapy.

The prospects for the future of pyran therapy seem high. The extreme toxicity noted by Regelson et al. was probably related to an excessive dose relative to the known LD_{10} in animal models. Defined fractions of pyran are now available: MVE-2, 3, and 4 are highly active in tumor models and have limited toxicity (*68*). Of a highly defined and restricted molecular weight range, they certainly should be investigated in phase I trials and, if doses which induce IF and activate macrophages can be determined, the fractions should then be subjected to phase II clinical trials either alone or in combination with chemotherapy or other modalities of treatment.

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GLUCAN

Glucan, the polysaccharide extract of zymosan with a mol wt of approximately 6,500, is a long-chain polyglucose with a 1-3 glucosidic linkage. It is a particulate, insoluble but digestible substance. Glucan is relatively nontoxic and has been found to be a potent RES stimulator and macrophage activator and an inducer of increased resistance to infection and antitumor activity in various experimental systems. Secondary effects include augmentation of the antibody response and augmentation of immunity mediated by T-cells, including allograft and xenograft rejection and augmentation of the GVH reaction with the use of donor spleen cells from glucan-treated animals. The great bulk of the glucan work has been done by DiLuzio and co-workers.

Antitumor activity has been demonstrated in a number of systems including the Shay myelogenous leukemia in the rat (*1*) in which glucan is active alone or combined with tumor cell immunization. Glucan has been associated with tumor regression in the syngeneic mouse adenocarcinoma DW10232 (*2*), in the melanoma B16 (*3*), and in S180 (*4*). In the melanoma B16, multiple doses administered from day 1 caused tumor regression in both normal and T-cell-deprived nude mice (*5*).

Glucan has also been active when combined with either chemotherapy or radiotherapy. Thus it is synergistic with CPP in the treatment of the Shay leukemia (*6*) and with BCNU chemotherapy in AKR leukemia (*7*). In the latter instances, neither BCNU nor glucan alone resulted in any long-term survivors, whereas appropriate combinations of BCNU and glucan led to 40% long-term survivors. With radiotherapy, it showed synergistic activity against locally inoculated and subsequently irradiated 6C3HeD lymphoma (*7*).

Not all studies of glucan have yielded positive results. Thus in 1 study, 2 carcinogen-induced fibrosarcomas failed to respond to glucan (*7*). In another, 1 batch of glucan showed antitumor activity to a mouse fibrosarcoma, whereas 2 other batches had no antitumor activity whatsoever (*8*). Finally, in another investigation, 5 syngeneic model tumors including line 10 hepatoma in the guinea pig, melanoma B16 in the mouse, mouse mammary carcinoma, and 2 mouse fibrosarcomas showed no response to different doses, routes, and schedules of glucan (*9*). These negative results cast serious reservations on the validity of the positive studies with glucan and suggest that more intensive study will be necessary before the widespread clinical investigation of this agent is encouraged.

Another area of biological activity of glucan has been protection from infection. *S. aureus* septicemia (*10*) and disseminated experimental candidiasis (*11*) are reduced in severity and show decreased mortality by treatment before but not after with glucan. Furthermore, glucan treatment reduces the increased susceptibility to infection induced in mice by CPP (*12*) or alcohol exposure (DiLuzio NR, Williams DL: Submitted for publication). Fungal infection with *C. neoformans* is also modified by glucan treatment (*13*). Intralesional therapy can reduce the severity of fungal skin infections (*13*).

Antitumor clinical studies with glucan have been done only to an extremely limited extent. Mansell and co-workers (*14, 15*) treated 40 patients with cutaneous and subcutaneous neoplasms, mainly metastatic malignant melanoma, with intralesional injections of up to 100 mg glucan and reported that almost all the injected tumor nodules regressed (68 of 70). On biopsy, macrophage infiltration was noted. Noninjected nodules regressed in only 2 patients. Israël and Edelstein (*16*) gave repeated injections of glucan into the cutaneous and subcutaneous tumor nodules of 11 patients with doses up to 180 mg/lesion and observed regression in 4 patients. Systemic therapy with glucan has not been used clinically. However, after intralesional treatment, fever and chills have been noted in addition to local inflammatory reactions and local pain.

Toxicity in the animal models has been mild; it includes sensitization to endotoxin (*17*) as well as local inflammatory and sometimes local ulcerating reactions.

Extensive immunological and related host defense research has been done in several systems. Thus the systemic iv or ip inoculation of glucan into experimental animals results in accelerated particle clearance; [^{131}I]RES test lipid (*18*), erythrocytes (*19*), and polymeric plutonium (*20*) have been investigated. The half-time is reduced approximately 50%, and appropriate studies indicate localization in liver and spleen. A prominent feature of iv or ip glucan administration is macrophage activation. In various tumor systems, macrophage phagocytic activity (*21*) and tumor cell inhibition or cytotoxicity are augmented as measured by [^{3}H]dThd incorporation (*22*), tumor cell colony inhibition (*23*), or ^{51}Cr release (*24*). The increased particle clearance and macrophage activation correlates well with increased serum lysozyme levels; some researchers (*25*) have proposed that serum lysozyme is an excellent and accurate measure of RES and macrophage activation in glucan-treated animals.

Glucan also affects lymphocyte-related components of the immune system. It increases the mitogen response to both the B-cell mitogen endotoxin and the T-cell mitogen Con A after a single dose (*26*). However, one should note that, after multiple doses of glucan, these mitogen responses are actually diminished. The primary and secondary antibody responses to SRBC are augmented by a single dose of systemically administered glucan (*27*). In addition, such T-cell activities as the GVH (*28*) and skin allograft rejection (*29*) are also augmented by a single dose of glucan.

In summary, glucan, a digestible particulate, high-molecular weight polysaccharide, is a potent macrophage and RES stimulator which has both infection protection and antitumor activity. However, controversy exists as to the degree and reproducibility of antitumor activity in animal systems; also, variations in batches may be a major problem. Therefore, more extensive trials with glucan in various appropriate tumor models by several investigators are indicated before this material is introduced into clinical trials.

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SECTION VI

Human Cancer Antigens

All attempts at modification of the immune response to human cancer are based on the assumption that cancer cells can be distinguished from their normal counterparts by the presence of distinctive cell surface antigens. As the most compelling evidence for the existence of such antigens has come from work in the mouse, a brief survey of current knowledge with regard to tumors and leukemias of the mouse is in order.

ANTIGENS OF TUMORS IN EXPERIMENTAL ANIMALS

The demonstration that sarcomas induced by polycyclic hydrocarbons, such as MCA, expressed strong transplantation antigens capable of causing tumor graft rejection in mice of the same inbred strain represents the cornerstone of the field of tumor immunology (*1, 2*). Later studies of tumors induced by oncogenic viruses, such as polyoma virus, showed that these tumors were also characterized by cell surface antigens that stimulated resistance to tumor grafts in syngeneic mice (*3, 4*). A striking difference was found between chemically induced sarcomas and virus-induced tumors: Each such sarcoma had individually distinct antigens, whereas different tumors induced by the same virus shared the same antigens (*5, 6*). Immunization with a chemically induced sarcoma brought about resistance to grafts of that tumor but generally no other, even when different sarcomas resulted from use of the same carcinogen in the same mouse. On the other hand, each tumor induced by an oncogenic virus elicited resistance to grafts of any other tumor induced by that virus. Although this distinction between sarcomas and tumors induced by chemicals and viruses, respectively, has been demonstrated many times, it is not absolute; some virus-induced tumors have had antigens which do not cross-react, and some chemically induced tumors appear to share cross-reacting antigens.

To learn more about the chemical structure and genetic origin of these tumor-specific antigens, researchers have directed much work toward defining them by in vitro techniques; these efforts are most advanced in virus-induced tumors. In tumors induced by oncoviruses, viral structural components incorporated into the cell surface are generally considered to represent the transplantation antigens. In contrast, tumors caused by DNA viruses, such as polyoma virus or SV40, do not express viral structural components. It appears that a virus-determined non-structural component, the T-antigen, is expressed on the cell surface and is responsible for the immunological rejection of these tumors. The study of the individually distinct antigens of chemically induced tumors is much less advanced because antibody detecting these antigens is found only rarely in immunized animals. Only recently has some progress been made in their serological analysis with hyperimmune sera (*7, 8*) and their biochemical characterization (*9*). The hybridoma technique for production of monoclonal antibodies promises to provide us with additional reagents for defining the surface antigens of chemically induced sarcomas.

For two reasons, knowledge of cell surface antigens of tumors of the mouse is most advanced in mouse leukemia (*10*): 1) Leukemia cells can be studied in cell suspension which facilitates serological analysis. 2) Because most mouse leukemias arise in the thymus, an easily accessible normal cell population (thymocytes) is provided and can be compared with the leukemia cell population. Several categories of surface antigens can be distinguished on mouse leukemia cells. Some antigens are found on both normal thymocytes and leukemia cells; they include antigens coded for by the H-2 complex, i.e., T-cell differentiation antigens such as Thy-1 and Lyt. Another system of antigens, called TL, shows the characteristics of leukemia-specific antigens in certain strains and normal differentiation antigens in other strains. They are restricted to normal thymocytes in certain mouse strains that express them and are referred to as TL-positive strains. The thymocytes of other strains (TL-negative strains) normally do not express TL antigens. However, leukemias may express TL antigens, even when they arise in mice of TL-negative strains. This anomalous appearance of TL antigens in leukemias of TL-negative strains indicates that all mice possess the structural genes for TL, but this information is normally repressed in TL-negative strains. During leukemogenesis, the normally silent *TL* locus in TL-negative strains is depressed, and this results in the appearance of TL-positive leukemia cells. A similar mechanism appears to be involved in the expression of another category of mouse leukemia cell surface antigens which are specified by the murine leukemia virus. Frequently, these antigens appear in leukemias of mouse strains that normally do not express them. Expression of fetal antigens or inappropriate H-2 antigens has also been ascribed to activation of normally silent genetic information. Further work is needed, however, before this possibility is established.

In the strictest sense, antigens specific for mouse leukemia cells (i.e., restricted to leukemia cells and never found on any normal cell) have not been found. What have been detected are antigens, such as TL and certain murine leukemia virus-related ones, that appear as normal differentiation antigens in some mouse strains and as TSA in strains that do not express them during normal life. Apparently, the coding genes are universal in the mouse, but in some strains they are never activated. Malignant transformation leads to expression of these normally silent genes and, because the appearance of the gene products is restricted to tumor cells in these strains, they have the characteristics of transformation-specific products.

Not all tumors of experimental animals are immunogenic in transplantation experiments. These examples of nonimmunogenic tumors have caused some investigators to raise serious questions about the general validity of the immunosurveillance concept and thus the whole field of cancer immunology. However, lack of immunogenicity in transplantation experiments does not necessarily imply that TSA are absent. Rather, it may be related to the complex interactions involved in immunological recognition and rejection by the host. An array of escape routes has in fact been identified by which tumors of known antigenicity can escape immunological destruction. Rather than argue the merits or deficiencies of the immunosurveillance concept, it seems more important for us to test the central hypothesis of the field, which states that

changes in cell surface antigenicity are an invariable consequence of the transition to the malignant phase, whether or not these antigens lead to rejection. To this end, serological techniques continue to offer the greatest promise because they are most precise. The possibility must be kept in mind that certain antigens may not elicit the production of antibody and their detection may thus depend on methods that measure cellular immunity.

ANTIGENS OF HUMAN TUMORS

Numerous authors have addressed the question of tumor-specific cell surface antigens of human cancer. A general impression exists that TSA have been demonstrated in many types of human cancers. This conclusion is not justified, however, and the existence of such antigens must still be considered uncertain. Specificity is the critical issue, of course, and a definition of the specificity of serological or cell-mediated immune reactions is made more easily in the mouse than in man. The difference is in the availability of inbred strains which permitted the transplantation studies that established the existence of distinctive cell surface antigens in tumors of experimental animals. The serological definition of these antigens also depended on inbred populations to provide the necessary reagents. In the absence of these advantages, the human cancer serologist is still attempting to develop approaches that can cope with the issue of specificity.

Most of the current knowledge of cell surface antigens of human cancers is derived from studies in which heteroimmune or allogeneic sera and lymphoid cells were used. However, apparently TSA defined by heterologous sera have always resulted, on more extended analysis, to be differentiation antigens, that is to say antigens characteristic of normal cells at some phase of differentiation. Tumor antigens detected by reactions with human sera fall into several categories. Some are alloantigens, particularly products of the HLA complex and *ABO* locus. Another category of antigens is related to the EBV. Seroepidemiology has not only proved the association between EBV and Burkitt's lymphoma (*11*) but also revealed the link between EBV and nasopharyngeal carcinoma (*12*) and infectious mononucleosis (*13*), associations that were totally unexpected. One difficulty in the interpretation of results of studies involving sera or lymphoid cells from one individual and tumor cells from another is the unknown participation of alloantigens in the reactions observed. It is for this reason that most surveys for CMI or humoral immunity to cell surface antigens of human cancer cells have not permitted distinction of tumor-specific reactions from those directed to other categories of antigens. The approach of autologous typing eliminates some of the difficulties involved in the determination of whether cancer patients recognize TSA on the surface of their cancer cells. It has been applied to the study of cell surface antigens of malignant melanoma (*14–17*), acute leukemia (*18*), astrocytoma (*19*), and renal cancer (*20*). Inasmuch as the work is most advanced in malignant melanoma, this tumor may serve to illustrate the classification of cell surface antigens that has emerged from autologous typing and the directions that can be followed in the further investigation of these antigens.

Serological Definition of Cell Surface Antigens of Malignant Melanoma by Autologous Typing

Essential features of autologous typing are 1) use of established tissue culture lines (rather than uncultured tumor cells of primary cultures) which will permit repeated serological testing, 2) restriction of direct serological tests to serum and tumor cells from the same individual (autologous combinations), so that the contribution of alloantibodies is eliminated and antigens that belong to the individually distinct category are detected, 3) use of several serological techniques that will reduce the possibility that antibody of a particular immunoglobulin class might be missed, and 4) extensive use of adsorption tests so that the occurrence of antigens on cells from various sources can be determined. Among these cells are cultures of as many different normal cell types as possible from the tumor donor, including skin fibroblasts, EBV-transformed B-cells, T-cells, and skin epithelium. These cultured autologous normal cells and noncultured peripheral blood cells, as well as allogeneic and xenogeneic normal and malignant cells, represent an extensive panel of absorbing cells used in the analysis.

Autologous typing of sera from 75 melanoma patients showed IgG or IgM antibody in 56 of them (*21*). Absorption analysis showed that three classes of melanoma cell surface antigens can be defined by autologous antibody. Class I antigens are individually distinct antigens and show an absolute restriction to autologous melanoma cells. Class 2 antigens are shared melanoma antigens, expressed not only by autologous melanoma cells but also by some allogeneic tumors. Class 3 antigens are not restricted to melanoma cells; they are expressed on an extensive range of normal and malignant cells of human and animal origin. In 30 patients whose antibodies have been sufficiently analyzed, the antibodies detected Class 1 antigens in 4, Class 2 in 5, and Class 3 in 21.

One of the questions raised by these studies is why so few melanoma patients have antibody to Class 1 or 2 antigens. Although it is possible that a lack of serological reactivity implies the absence of immunogenic tumor antigen, this is not necessarily so. Alternate explanations are that serological assays may not be sufficiently sensitive or the immunological recognition of tumor-specific melanoma cell surface antigens may be restricted to cellular immunity. In addition, the melanoma cell population could reflect immunoselection for cells with low antigenic expression. As the tumor progresses, fewer immunogenic clones would replace more immunogenic populations. Much more information is required regarding changes in surface antigenic phenotype related to tumor progression. In addition, outgrowth in tissue culture, essential for autologous typing, could select melanoma cells with low antigen expression from an originally mixed population. Undetectable reactivity to Class 1 and 2 antigens could also be due to complexing of antibody with antigen or anti-idiotype antibody. The finding of immune complexes in patients with melanoma, perhaps relating to shedding of antigen by

tumor cells, raises that possibility (*22*). Lewis et al. (*23*) have reported that melanoma patients form anti-idiotype antibodies and that they may be the reason for the lack of detectable antibody in melanoma patients with disseminated disease. Genetic factors may provide another reason for the lack of a humoral immune response to melanoma cell surface antigens. In experimental systems, the ability of animals to respond to a variety of antigens including certain tumor antigens is controlled by immune response genes. Lack of humoral immunity to Class 1 and 2 antigens in most of the melanoma patients could reflect a genetically determined inability to respond to these antigens. Investigation of this possibility awaits definition of the genetic determinants of the immune response in man.

Changes in Serological Reactivity During the Course of Melanoma

The finding that patients with malignant melanoma may have antibodies to cell surface antigens of their tumor cells raises the following questions: 1) Does the antibody titer remain constant or change with time? 2) Is a change in antibody titer detected by one assay paralleled by a similar change measured by another? 3) Do different assays detect antibodies of the same specificity? 4) Does the specificity of antibodies in the serum of a given patient change with time? 5) Do cell lines derived from different metastases of the same patient express the same Class 1 or 2 antigens? Only rudimentary information regarding these questions is available at the present time. Scientists will need to study the time course of a series of melanoma patients to determine whether the presence or absence of reactivity to Class 1 or 2 antigens is of prognostic significance and if changes in serological reactivity and in the clinical course are related.

Biochemical Characterization of Melanoma Cell Surface Antigens

Now that melanoma cell surface components have been defined by serological methods, several biochemical techniques can be applied for isolation and characterization of the antigens. Radioimmunoprecipitation is particularly powerful; it has been used extensively in the study of antigens coded for by the MHC in humans and in the mouse. This technique has been used successfuly in the identification of HLA products, β_2-microglobulin (*24*), Ia (DR) antigens (*25, 26*), and fibronectin (*24*) on the surface of melanoma cells. The expression of Ia antigens and melanoma cells is variable, with some melanoma culture lines expressing high levels of Ia and others expressing little or none. Normal melanocytes appear to express little or no Ia antigen (*27*). The absence of Ia on mature melanocytes and its presence on melanoma cells, possibly derived from less differentiated cells of the melanocyte series, are paralleled by a progressive loss of Ia expression during differentiation of B-lymphocytes and cells of the myelocytic series (*28, 29*). Perhaps typing of melanoma cells for Ia antigen expression will provide information regarding the stage of differentiation at which malignant transformation occurred. Whether expression of Ia antigen on melanoma cells correlates with the biological behavior of the tumor remains to be determined.

In the analysis of Class 1 or Class 2 melanoma antigens detected by autologous typing, immunoprecipitation techniques have not been useful, possibly because antibody titers were too low. An alternative approach is for one to solubilize melanoma antigens, fractionate them by standard biochemical techniques, and monitor the fractions for specific antigens by inhibition assays with human sera detecting Class 1 or 2 antigens. One of the Class 1 antigens has been partially characterized with the use of this approach. It was solubilized by limited papain digestion and found to be a glycoprotein with a mol wt in the range of 25,000–40,000. The solubilized antigen was not related serologically to HLA, Ia, or β_2-microglobulin (*30*).

Genetic Origin of Melanoma Antigens

One of the fundamental questions about Class 1 and 2 melanoma antigens is whether they and the genetic events that lead to their expression are causally linked to malignant transformation or whether they are "luxury" products of the cell, the result of epiphenomena that can be lost without effecting the neoplastic state. The genetic origin of individually distinct tumor antigens is as much a mystery at present as it was when these antigens were first discovered as transplantation antigens in chemically induced tumors of the mouse. Several possibilities have been considered that account for their extraordinary polymorphism, including mutational events involving structural or regulator genes, relation to preexisting antigenic variability in the normal cell population, relation to oncogenic viruses (there is growing evidence that murine leukemia viruses are highly polymorphic). Epigenetic explanations have also been put forward: One idea is that an altered surface display of gene products might result in novel antigenic determinants without any need for primary genetic change. Chromosomal mapping of Class 1 antigens of human melanoma cells may be possible by the technique of somatic cell hybridization. Fusion of Chinese hamster cells with melanoma cells expressing a Class 1 antigen has resulted in hybrids expressing that antigen; results obtained in an extensive analysis of one hybrid series indicated that the antigen is coded for by a locus on chromosome 19 (Resnick LA, Pravtcheva DD: Unpublished observations). Further studies should show whether these individually distinct melanoma antigens represent a family of polymorphic but structurally related molecules coded for by a single locus or totally unrelated molecules coded for by many loci.

Detection and Characterization of Melanoma Cell Surface Antigens With Monoclonal Antibodies

The development of the hybridoma methodology has added a new dimension to the serological analysis of human cancer cells. Monoclonal antibodies, produced by the hybrid progeny of a myeloma cell and a normal immunoglobulin-secreting B-cell, are likely to replace, for many purposes, conventionally prepared antisera, and thus allow the preparation of highly specific reagents in large quantity. Several technical problems remain to be resolved, such as improvement of selection methods for isolation of

the desired clone and reduction of clonal instability due to chromosomal loss. However, application of the methodology in its present form has already produced monoclonal antibodies to histocompatibility and differentiation antigens of man, mouse, and rat. In the cancer field, several groups have produced hybridomas that secrete monoclonal antibodies to human cancer cells and fuse their spleen cells with mouse myeloma cell lines. Again, the work in malignant melanoma may serve as an example (*31–41*). Various systems of melanoma cell surface antigens have been defined with monoclonal antibodies, each with a characteristic cellular distribution, and current efforts are directed at characterization of these antigens in biochemical terms. The antigenic system with perhaps the most restricted distribution is a glycolipid antigen found on melanomas, astrocytomas, and melanocytes but not on epithelial cells, fibroblasts, or cells of hematopoietic origin (*35*). However, none of the antibodies developed so far recognize cancer-specific cell surface antigens. Mouse monoclonal antibodies can only tell us, of course, what the murine immune system recognizes on human cancer cells. As human myelomas are now becoming available for fusion with human lymphocytes, it will be possible for investigators to produce human monoclonal antibodies and define more precisely the cancer cell surface antigens that can be detected by the human immune system. As a consequence of the intense activity in this area, we can look forward to a comprehensive picture of the surface antigenic structure of melanoma cells and other human cancer cells in the near future. This work may eventually lead to new classifications of tumors, once correlations of certain clinical features with the presence or absence of cell surface antigens detected by these antibodies have been established. Apart from being powerful analytical probes, monoclonal antibodies may also find important applications in diagnosis and therapy.

Cell-mediated Immune Reactions to Melanoma Cell Surface Antigens

The literature dealing with cell-mediated immune reactions to human cancer cell surface antigens is extensive, and again melanoma takes a prominent place (*42, 43*). Allogeneic combinations of lymphocytes and target cells have been used in most of these studies. In this setting, the demonstration of TSA is extremely difficult just as it is when allogeneic combinations of sera and target cells are tested in serological studies. The use of autologous combinations of lymphocytes and target cells is required not only for exclusion of reactions to normal alloantigens but also for detection of individually distinct cell surface antigens. Even if shared TSA are involved, autologous testing may be critical to the demonstration of their specificity.

One of the important findings regarding T-cell cytotoxicity in the mouse is that killing of various infected or chemically modified target cells by T-cells requires that effector and target cells share the D- or K-end of the *H-2* locus, a phenomenon that is referred to as H-2 restriction (*44, 45*). This H-2 restriction has also limited cell-mediated cytotoxicity for tumor antigens in some murine tumor systems (*46*). Whether HLA restriction is also characteristic of cell-mediated cytotoxicity for TSA of human cancers remains to be seen. If this is true, autologous typing would again be preferable because it is technically far easier for one to accomplish than is HLA matching for allogeneic effector cells and target cells to assure the requisite compatibility. Even when studies of lymphocyte cytotoxicity in patients with malignant melanoma were restricted to autologous combinations of effector and target cells, definition of the specificity of observed reactions has been extremely difficult (*47*). The problem is that methods, such as competitive inhibition assays and immunoadsorption, that are valuable in determining the specificity of cell-mediated cytotoxicity reactions in cell suspensions are not suitable for work with monolayer cells.

The discovery of TCGF which permits the continuous growth of primed human T-lymphocytes opens new ways for testing cell-mediated cytotoxicity reactions to human tumors (*48*). The T-cell cultures maintained in media containing TCGF (IL-2) maintained cytotoxic function and antigenic specificity (*49, 50*). Current efforts are directed toward the determination of whether cloned T-cells from cancer patients will be specific and effective probes of human cancer antigens eliciting cellular immunity.

Development of Immunogenic Human Cancer Vaccines

Now that a range of human cancer cell surface molecules has been defined by serological analysis, one of our current challenges is to determine which of them are or can be made immunogenic in man. Whereas some of the antigens are known to elicit an immune response in the autologous host and serological methods to monitor that response and define its specificity are available, the determination of whether cancer patients can be immunized with these antigens is justified and timely. The idea of a human cancer vaccine is, of course, not new; many cancer patients have been given injections of autologous or allogeneic tumor cell preparations over the past 50 years. However, the complexity of these studies makes an assessment of the value of this approach to cancer therapy impossible. The testing of human cancer vaccines is fraught with difficulties and uncertainties ranging from the appropriate type, dose, route, and frequency of vaccination to suitable patient selection and to a critical evaluation of the clinical response. With all these variables, an analysis of the effectiveness of different vaccines by evaluation of clinical response alone would be extremely time-consuming, and the picture concerning the value of a cancer vaccine may not be any clearer than that which has emerged from past studies. A basic problem has been our lack of rapid and precise methods to monitor the persistence of relevant antigens during vaccine construction and to assess the immunogenicity of these antigens in patients receiving the vaccines. With regard to vaccines against infectious diseases, serological responses to bacterial or viral antigens have been an essential step in their development. The lack of comparable serological tests with which we could monitor the effectiveness of cancer vaccines has been a major impediment to investigating this approach to cancer therapy. Now that autologous and allogeneic typing

systems have been developed for definition of cell surface antigens of melanoma and other cancers, serological tests are available which can gauge the immunogenicity of cancer vaccines. With these methods, testing the immunogenicity of different types of vaccines sequentially in small groups of patients is possible, thus vaccines of demonstrated immunogenicity can be tested for therapeutic efficacy.

Preliminary results of early trials of this type are now available. In the first study, the vaccine was prepared from irradiated cultured autologous melanoma cells. The reason this type of vaccine was chosen came from the restriction of Class 1 antigens to autologous melanoma; if these antigens are important in the immunological control of cancer, autologous vaccines are a logical way for one to proceed. However, serological analysis showed that vaccinated patients responded only rarely to this type of vaccine (*51*). As a next step, vaccines prepared from allogeneic melanoma cells expressing cross-reacting melanoma cell surface antigens were tested. The serological definition of systems of shared melanoma antigens provides the rationale for the use of allogeneic melanoma vaccines and the means by which the appropriate allogeneic cell line to be used is selected. An advantage to the use of allogeneic vaccines comes from the theoretical possibility that foreign determinants present on the allogeneic cell surface may facilitate or help immune recognition of TSA (*52*). Serological analysis showed again, however, that a response to shared melanoma antigens was induced only rarely, although high titers of antibodies against HLA antigens were induced in almost every patient (*53*).

Another approach that merits investigation is vaccination with melanoma cells infected with virus. This approach is based on the finding that the immunogenicity of tumor antigens appears to be augmented following infection of tumor cells by certain viruses, particularly myxoviruses. Homogenates prepared from tumors infected with virus (viral oncolysates) were more effective than comparable preparations of noninfected tumor cells in inducing transplantation immunity (*54–56*). Viruses that are being investigated in the preparation of viral oncolysates of melanoma cells are the vesicular stomatitis (*57*) and vaccinia (*58*) viruses. Prager and Baechtel (*59*) proposed several methods to augment the immunogenicity of tumor cells by direct chemical or enzymatic (e.g., neuraminidase) modification of the cell surface. As an example, a PPD coupled to cells of a transplantable MCA-induced sarcoma increased the immunogenicity of the tumor cells in syngeneic mice that had preexisting delayed hypersensitivity to BCG (*60*). Because BCG has been widely used in clinical immunotherapy, this method of augmenting the immunogenicity of tumor cells might have particular clinical appiicability.

The presentation of cancer antigens is not the only factor that determines the immunogenicity of cancer vaccine. Progress in basic immunology has revealed mechanisms by which the recipient's immune system rather than the immunizing agent determines the response to immunization. One of them is based on genetic factors. Immune response genes determine whether an individual's immune system can recognize a given antigen; at the present time, nothing is known about immune response genes that govern the immune response to human cancer antigens. Another mechanism that controls the magnitude and duration of immune response resides in the balance of activity of the regulatory networks of the immune system. Eliciting a maximal response to cancer vaccines may well depend on development of strategies by which genetic restriction of the immune response can be overcome and the balance of immunoregulation forced in the direction of induction rather than suppression. The latter has already been attempted experimentally in various ways: administration of antibodies thought to be specific for suppressor cell surface antigens, chemical modification of antigens to change the balance of the response in favor of inducer cells over suppressor cells, and chemotherapy or irradiation to reduce the suppressor cell population. Although the effectiveness of any of these approaches is uncertain at this point, undoubtedly, investigators who attempt immunization with cancer antigens will have to take into account host factors that regulate the response to these antigens.

No doubt serological methods now offer us the most precise and versatile tools to test the immunogenicity of cancer vaccines; an optimal antibody response to unique or shared cancer antigens is a valid criterion by which vaccines can be selected for therapeutic trials. There is, of course, a general impression that increased cellular rather than humoral immunity is the desired end point of immunological manipulation in cancer patients. However, we know too little about this to form a judgment in the matter. Concern has also been expressed that immunization may result in augmented rather than restricted tumor growth, particularly if the procedures used induce increased humoral immunity. Again, we do not know if this potential exists in man; enhancement of tumor growth has not been observed in any patients who received cancer cell vaccines (or, for that matter, in any animal experiment with strictly syngeneic tumors). We will have to remain alert, nonetheless, to this possibility.

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SECTION VII

Lymphocytes for Cancer Therapy

INTRODUCTION

Inasmuch as the critical immunological reaction responsible for tumor rejection in animal models has been shown to be mediated by immune cells reactive to TAA, scientists have made many attempts to eradicate established tumor by infusing the requisite effector cells. Although some of the theoretical asumptions which underlie this approach to therapy have been validated in animal models, the validity of similar assumptions in man is still being tested. The emphasis in this brief review, however, will not be on the assumptions but on what is known about the use of transferred cells as a form of adoptive tumor therapy in man and animals. Much of the information has been reviewed elsewhere (*1–6*).

Many animal models for adoptive therapy have been developed during the past 10–15 years. Concurrently, empiric attempts were initiated for treatment of humans with lymphocytes with inconclusive results. After a period of relative neglect, researchers have shown an impressive resurgence of interest in this investigational approach to therapy, largely stimulated by developments which make it more likely that adoptive therapy will eventually be used in patients. The resurgence is due especially to technological advances which make possible the sensitization of lymphocytes in vitro to alloantigens and tumor antigens and the generation and maintenance in vitro of large quantities of cells with specific antitumor reactivity for potential therapy. In addition, the area has been advanced greatly by increased knowledge of human and animal lymphocyte subsets and their interactions, as well as by clinical results of marrow transplantation for the treatment of hematologic neoplasms in man, especially the demonstration that a marrow allograft with GVH disease exerts a significant antileukemic effect in man, as predicted from animal models.

The clinical potential of adoptive therapy can only be evaluated by our developing effective animal models and using them to identify problems which must be resolved before adoptive immunotherapy can be applied to man. In this section, we review the results of adoptive therapy in animal models, discuss some of the apparent prerequisites for tumor eradication, and explore some of the problems posed by those prerequisites for potential application of adoptive therapy to humans. Empiric attempts already made clinically will also be summarized, and directions for future studies will be suggested.

USE OF SYNGENEIC LYMPHOCYTES

The most extensive studies have used syngeneic cells in the treatment of a host bearing a syngeneic tumor. The antitumor response can thus be dissected, and the mechanisms of immunotherapy can be determined without the immunological complications presented by transplantation of histoincompatible tissue. Moreover, some results may be directly extrapolated to clinical situations in which the cells to be transferred are syngeneic, i.e., from a genetically identical twin, or autologous, i.e., from the patient himself.

Although syngeneic cells immune to tumor can affect tumor growth when administered shortly before or after tumor challenge (*2*), they rarely affect a tumor once it has become established in a normal host (*1, 5*). The exceptions to this rule have been most impressive. Thus syngeneic cells from donors immunized to tumor in vivo have eradicated an established murine methylcholanthrene sarcoma (*7*), Moloney sarcoma virus-induced tumors in mice (*8–10*), and an established hepatoma with lymph node metastases in guinea pigs (*11*).

Cells for therapy have also been generated in vitro. Thus normal lymphocytes cultured with tumor cells for several days undergo proliferation and become cytotoxic to tumor cells in vitro. However, although such cells are cytotoxic to tumor in vitro and protective in a neutralization assay (*6, 12–14*), they have not been significantly and reproducibly effective therapeutically when used as the sole form of therapy in a tumor-bearing host (*12–14*). By contrast, cells primed to tumor antigen in vivo, i.e., cells from animals immunized once to the tumor and then secondarily sensitized to tumor in vitro, have been therapeutically effective in vivo against several tumors, such as a transplantable progressively growing Moloney sarcoma virus tumor (*15, 16*), FBL-3 (*17*), P-815 mastocytoma (*18*), and an L1210 leukemia (*18*).

The principal factors that limit the efficacy of adoptively transferred lymphocytes in a tumor-bearing host are the large tumor burden and the development of host suppressor cells and/or factors which interfere with the efficacy of infused donor cells. Thus infused cells are less effective when used either against a large tumor or after tumor inoculation (*3, 11*). Accordingly, transferred cells are far more effective when administered to animals whose suppressor cells have been decreased by preirradiation of the host (*15, 16, 18*) and/or by T-cell depletion of the host (*19*) than in normal tumor-bearing hosts. The suppressor T-cells generated in association with progressive tumor growth may explain why significant antitumor effectiveness by syngeneic cells transferred to a normal host bearing a clinically detectable tumor is rarely achieved (*19*).

Because other modalities of therapy such as surgery, radiation, or cytotoxic chemotherapy can potentially decrease both the tumor load and the suppressor cells, some scientists have used immune cells in animal models to destroy tumor cells specifically, in concert with other modalities of therapy. As an adjunct to surgery, immune syngeneic cells were effective against a syngeneic Lewis lung carcinoma (*20*). Barnes et al. (*21*) studied the use of cells with radiation therapy largely by treating tumor-bearing mice with supralethal doses of total body irradiation to destroy tumor cells and then infusing syngeneic marrow, with or without lymphocytes, to reconstitute both the hematopoietic and immunological systems of the host. Usually, this approach has involved cells from normal donors and has not been effective against established tumors (*22*).

The most positive and most reproducible results have been reported in models in which antigenic syngeneic advanced tumors were eradicated by a combination of nonlethal noncurative chemotherapy plus lymphocytes immune to TAA. This "adoptive chemoimmunotherapy" approach has been effective against several advanced syngeneic tumors (*3*), most notably Moloney (*6, 23*), L1210 (*24, 25*), Friend (*26, 27*), EL 4 (*6, 28, 29*), Rauscher (*30*),

and L_2C leukemias (*31*). In all those models, maximal immunotherapeutic efficacy required that the infused syngeneic cells be immune T-cells capable of proliferating in the host and of persisting in the host for some time (*6*). Cells from donors immunized to TAA are effective. Moreover, normal lymphocytes primarily sensitized to tumor in vitro (*28, 32*) or lymphocytes obtained from animals primed to TAA in vivo and then secondarily sensitized to tumor in vitro have also been effective (*17, 29, 33, 34*). As an adjunct to chemotherapy, cells primarily sensitized to tumor in vitro have been immunospecifically effective against EL 4 and Moloney leukemia (*32*). Cells secondarily sensitized to tumor in vitro have been most extensively studied for their demonstrated effectiveness against FBL-3 (*17, 33, 34*). Interestingly, their efficacy relative to that of primed fresh cells which were not cultured varied greatly and depended on the particular in vivo priming regimen used prior to culture (*33*).

The prerequisites for success of adoptive chemoimmunotherapy are still being studied. The identity of the donor effector cells, the influence of the host, and the role of chemotherapy are all critical.

Although various non-T-effector cells are cytotoxic in vitro, the critical effector cell in vivo in all the adoptive therapy models has been a T-cell immune to tumor (*10, 16, 19, 32, 35, 36*). Although the particular T-cell subsets active in various models are being studied, the lack of correlation between cytotoxic reactivity of effector cells in vitro and their antitumor activity in vivo (*33, 34*) suggests that the 2 assays reflect the actions of different T-cell subsets. The T-cells have been operationally separated by Lyt phenotypes with the Lyt 1^-2^+ cells containing cytotoxic and suppressor cells, Lyt 1^+2^- cells containing the amplifier, helper, and DTH effector cells, and Lyt 1^+2^+ cells containing precursors for the other T-cell subsets and some of the cytotoxic and suppressor cells (*37, 38*). In several models, tumor neutralization and prevention of outgrowth of a tumor by prior inoculation of immune cells required T-cells of the same Lyt phenotype as were required for cytotoxic reactivity against the same tumor in vitro (*39*). However, a recent study demonstrated that the predominant donor effector cell required for eradication of advanced disseminated FBL-3 by adoptive chemoimmunotherapy was an immune T-cell which was not cytolytic to FBL-3 in vitro (*36*). Thus the lymphocytes cytotoxic to FBL-3 in vitro were of Lyt 1^-2^+ and Lyt 1^+2^+ phenotypes, whereas in vivo the cell-mediating effector function in chemoimmunotherapy was Lyt 1^+2^- (*36*). In a rat model, in which T-cell subsets are less well characterized, the subset required for effective immunotherapy in vivo was also distinct from that required for tumor cytotoxicity in vitro (*16*).

The mechanism by which immune donor T-cells exert their antitumor effects has not been established. The possibility that it reflects largely direct tumor destruction by infused donor cells already cytotoxic is considered unlikely by the lack of correlation between cytotoxic reactivity in vitro and therapeutic efficacy in vivo and the demonstration that different T-cell subsets are involved in the two effector functions. It is more likely that the donor T-cells may exert an antitumor effect by direct cytotoxicity and by helping, amplifying, or recruiting other donor or host cells to destroy the tumor (*3, 20, 40, 41*). Thus although the effector cell ultimately responsible for the end results of therapy may be a tumor-specific, cytotoxic lymphocyte, an amplifier T-cell may be required for the continued generation of cytotoxic effeetor cells in vivo. This hypothesis is supported by the observation that after adoptive immunotherapy, there is a period of tumor growth before the tumor is ultimately eliminated (*16, 19, 42*), which suggests that some time is required for sufficient expansion of a pool of cytotoxic effector cells.

The host clearly influences the end results of adoptive therapy. For example, the peripheral blood of mice bearing progressively growing FBL-3 contains both FBL-3 cells and lymphocytes which could not prevent fatal growth of this leukemia in the primary host but which were curative when used as an adjunct for adoptive chemoimmunotherapy of it in a secondary host (*43*). Moreover, if Lyt 1^+2^- cells function in vivo largely by providing an amplifier function for induction of cytotoxic lymphocytes, then the host must be capable of providing the necessary precursors of cytotoxic lymphocytes. A negative host influence is suggested by the observation that infused donor cells are more effective in tumor-bearing hosts who have been preirradiated (*15, 18*), rendered T-cell deficient (*19*), or pretreated with CPP (*44*) so that the suppressor T-cells which interfere with the effectiveness of donor cells are eliminated.

The chemotherapy in syngeneic adoptive therapy has several important functions. It can decrease the tumor load (*42*), act against immunosuppressive host factors (*44–46*), possibly affect tumor antigenicity (*47*), and increase the susceptibility of tumor cells to immune-mediated lysis (*48*).

Effective adoptive immunotherapy requires large numbers of effector cells. These may be generated best by sensitization of lymphocytes primarily or secondarily in vitro. Primary in vitro sensitization makes normal cells immune without exposing the normal donor to tumor. Secondary sensitization in vitro can generate large numbers of effector cells or effector cells with an enhanced antitumor activity and, in addition, represents a way to bypass those mechanisms which limit the generation and/or expression of the antitumor effector cells in the tumor-bearing host, such as immunosuppression of tumor antigens, blocking factors, or suppressor cells (*49–51*).

The use of lymphocytes primarily sensitized in vitro for antitumor therapy in vivo is receiving increasing attention. Preliminary results are encouraging (*32*).

When used alone or in combination with other therapies, lymphocytes secondarily sensitized to tumor in vitro have been significantly and immunospecifieally therapeutically effective against established tumors (*6*). Three major problems have been noted. The first is a lack of correlation between assays for different antitumor activities, so that in vitro assays cannot be used as a refleetion of the relative success of the sensitization methods or to predict in vivo efficacy (*33, 34*). The second problem is the generation of the wrong cell. Thus, under certain conditions, suppressor cells can be generated in culture, which, when administered in vivo, can interfere with the therapeutic efficacy of concurrently administered immune cells in adoptive chemo-

immunotherapy (*52*). The results emphasize the need for the study of ways the requisite antitumor effector cells can be generated preferentially rather than the undesired suppressor cells.

The third problem is the limited quantity of effector cells generated and maintained in culture. Under standard conditions of in vitro sensitization, insufficient expansion of effector cells occurs. The principal approach to resolution of that problem has been the use of the TCGF, IL-2 (*53*). In the presence of IL-2, activated T-cells are induced to proliferate and can be maintained continuously in culture without the continued presence of antigen (*54*); T-cells thus maintained have been reported to be cytolytic in vitro (*55, 56*), and, most recently, to be immunospecifically effective in adoptive chemoimmunotherapy of FBL-3 and EL 4 (*57*). The therapeutic results are still sparse and may be complicated by two possibilities: The IL-2-dependent, long-term cultured lymphocytes may require the continual presence of IL-2 in the host to retain their therapeutic efficacy, and 2) such long-term cell lines may exhibit different homing characteristics which might affect their therapeutic efficacy (*58*). The former possibility is supported by the recent observation (*59*) that the antitumor chemoimmunotherapeutic effectiveness of donor lymphocytes, which had been primed in vivo, secondarily sensitized to tumor in vitro, and numerically expanded by long-term culture with IL-2, was enhanced by the administration of purified IL-2 to the tumor-bearing recipient.

USE OF ALLOGENEIC LYMPHOCYTES

The use of allogeneic lymphocytes for therapy is far more complex and presents additional issues not posed by syngeneic cells. These include the rejection of donor lymphocytes by the host, the induction of the GVH reaction or disease by donor cells, and the potential effect of GVH disease on the tumor to be treated.

Although allogeneic lymphocytes can protect animals against a subsequent tumor challenge, such cells have rarely been effective when administered after tumor inoculation (*2, 5*). The failure of allogeneic lymphocytes to destroy established tumor in the normal host is probably due to their failure to persist in the host for some time (*4*), to the large tumor burden which such cells encounter when used alone, and to the generation of putative host suppressor factors (*51*).

All three problems may be overcome by the use of allogeneic lymphocytes as an adjunct to chemotherapy and/or radiation administered in doses sufficiently cytotoxic to reduce the tumor load and sufficiently immunosuppressive to prevent or delay graft rejection. This approach is best represented by the use of marrow transplantation after supralethal chemotherapy or chemoradiotherapy (*4*). Allogeneic marrow transplantation in leukemic mice has been associated with significant and often fatal GVH disease which researchers have found difficult to control but which also exerted an antileukemic effect (*22, 60-63*). Early treatment of GVH disease interfered with its antitumor effect (*64*). The problems encountered in animal models have now been observed in marrow transplantation in man (*65*).

In allogeneic adoptive chemoimmunotherapy, doses of chemotherapy and/or radiation sufficiently suppressive to prevent rapid rejection of donor lymphocytes by the host tend to be associated with GVH disease. Nevertheless, allogeneic lymphocytes have been used successfully as an adjunct to noncurative nonlethal chemotherapy to treat Moloney leukemia (*23, 28, 66, 67*), L1210 (*25*), Moloney sarcoma (*9*), a Gross virus leukemia (*68, 69*), and a rat sarcoma (*70*).

Donor cells reflecting variable degrees of histoincompatability with the host have been tested in adoptive chemoimmunotherapy for their antitumor efficacy, ability to induce GVH disease, and for the ability of the GVH disease to exert an antitumor effect. The results have not been conclusive.

Lymphocytes from donors compatible at the MHC have no therapeutic effect against established tumors unless preimmunized to tumor and can occasionally, though not predictably, cause significant GVH disease (*23, 25, 66, 67, 69, 71*). In an effort to obtain immune cells which might be effective against tumor but might not cause GVH disease, Martin and co-workers (*72*) explored an approach recently based on the observation that tumor cells can express genetically inappropriate or "alien" histocompatible antigens, and others (*73*) found that cells primed with pooled alloantigens can become cytotoxic to tumor targets. Therefore, the possibility was considered that such cells sensitized to alloantigens may be sensitized to cross-reactive antigens present on leukemia target cells as well. Thus cells from CBA ($H\text{-}2^k$) mice alloimmunized in vivo against tumor and nontumor tissues of different H-2 composition exerted a significant graft-versus-tumor effect when transplanted into lethally preirradiated AKR ($H\text{-}2^k$) mice bearing an AKR leukemia and were not associated with increased GVH disease in a secondary host (*74*). However, the antitumor effect was documented only by bioassay in the secondary host (*74*).

By contrast to MHC-compatible lymphocytes, MHC-incompatible cells exert a significant therapeutic effect in animal models even if not immune and may become more effective if preimmunized (*4, 9, 67, 70*). Moreover, when infused in large numbers, they usually cause fatal GVH disease (*9, 75*).

Studies with allogeneic cells sensitized in vitro are too preliminary to permit conclusions. Their use is especially complicated by the problem of GVH disease. Approaches to control or prevention of GVH disease have met with only limited success in non-tumor-bearing animals and only rare success in tumor-bearing hosts (*4, 69*). The most recent approaches have involved a new immunosuppressive agent, cyclosporin A (*76*), total lymphoid irradiation of the host (*77*), and depletion of T-cells from donor cell suspensions (*71, 78*).

CONCLUSIONS FROM ANIMAL STUDIES

Effective models for adoptive therapy of advanced antigenic tumors in animals have been developed. The prime requirements for efficacy have been determined and some of the problems relevant to potential clinical application have been identified.

The most extensive studies have been performed in totally syngeneic models with cells immune to tumor. Although such cells can occasionally eradicate large tumors, their efficacy is limited by a large tumor load and by host suppressor factors, especially suppressor cells. Therefore, immune cells are most effective when used as an adjunct to radiation therapy and/or antitumor chemotherapy which can decrease both the tumor load and the suppressor cells. The donor effector cell critical for therapy has not been identified in all models, but, in the most intensively studied model, is an Lyt 1^+2^- cell which is not cytolytic in vitro but probably amplifies the cytotoxic T-lymphocyte response in vivo. The requisite effector cells for therapy in vivo can now be generated in vitro but are complicated by concurrent generation of suppressor cells which can interfere with the therapeutic efficacy of effector cells. Finally, immune lymphocytes can be maintained in long-term culture with IL-2 and can retain some in vitro reactivities and, in at least one model, significant therapeutic antitumor efficacy. The efficacy of cloned T-cell subsets is still being tested.

Allogeneic lymphocytes are rarely therapeutically effective when used alone but have been effective against established tumors in some models when used with radiation and especially chemotherapy. The cells should be immune to tumor and should persist and proliferate in the host for some time to be maximally effective against the tumor. These prerequisites emphasize the problem of the immunogens, the sensitization regimens, the need for immunosuppressive antitumor therapy to prevent rapid rejection of donor lymphocytes by the host and the resultant problem of potentially fatal GVH disease. However, GVH disease also has a significant effect against some tumors.

The results reviewed should stimulate investigators to: 1) develop additional animal models for adoptive immunotherapy against tumors of lesser immunogenicity and, of more recent origin, in the primary as well as in secondary hosts, so as to mimic more closely what is presumed to be the clinical situation; 2) identify the effector cells generated under different conditions for tumor eradication and study their interactions with other donor or host cells for better understanding of the positive and negative influences on their efficacy; 3) derive methods for sensitizing lymphocytes in vitro so that the requisite effector cells but no suppressor cells can be generated; 4) identify the effector cells maintained in long-term culture, clone the cells, study the interactions between cloned cells of different subsets and their products; 5) determine the optimal immunogens in vivo and in vitro, including TSA, differentiation antigens, alloantigens, or alien antigens; 6) prevent, and more importantly, control GVH disease and use it for its antitumor effectiveness, with emphasis on approaches to imparting preferential and/or specific antitumor activity to the GVH reaction; 7) determine the contribution of the tumor-bearing host to immunotherapy and the effects of chemotherapy and radiation on that contribution so as to derive optimal conditions for maximum effective immunotherapy.

STUDIES IN HUMANS

Many cancer patients have been empirically treated with lymphocytes with inconclusive results. However, the conditions were not consistent with the prerequisites derived from animal models. The trials have been reviewed in detail elsewhere (*2, 5*). Normal allogeneic lymphocytes have been given to cancer patients without convincing benefit (*2, 5*). Cells from allogeneic donors immunized with the patient's tumor material have been administered to many patients with some objective responses. Most attempts involved cross-immunization with killed tumor cells from patients with the same type of tumor followed by cross-transfusion of lymphocytes (*79–81*). Rapid rejection of donor lymphocytes by the preimmunized recipient would be expected. Six patients with various advanced cancers received lymphocytes from normal HLA identical donors and 2 were reported to respond, 1 in conjunction with chemotherapy (*82*). Patients with osteogenic sarcoma who had amputations have received lymphocytes from allogeneic donors immunized with the patients' tumor cells without significant effect (*83*).

On the assumption that lymphocytes from cancer patients have antitumor reactivity whose full expression is suppressed in vivo but which could be unmasked or enhanced by in vitro exposure to tumor cells or immunostimulants, such as PHA or BCG, autologous lymphocytes thus manipulated in vitro have been readministered to the patients (*84–86*) with inconclusive results (*5*).

Lymphocytes as an adjunct to other therapies in man have received too little attention. The approach is best represented by studies in which patients with hematologic neoplasms have been treated with supralethal doses of chemotherapy and total body irradiation plus their bone marrow or that from a syngeneic or allogeneic donor. The chief function of the infused marrow is hematopoietic restoration, but an immunotherapeutic antileukemic effect of donor marrow is also considered likely.

Patients with refractory acute leukemia and lymphoma have been cured by chemoradiotherapy and identical twin marrow (*87, 88*). The principal problem is leukemia relapse. It is not known whether the infused marrow exerts any antitumor effect; infusion of normal twin PBL after marrow transplantation did not influence the incidence of relapse in the small number of leukemia patients studied (*87*). Sensitization of donor cells prior to infusion has not been reported.

Allogeneic marrow transplantation from MHC-identical siblings is also complicated by a high incidence of leukemia relapse (*65*). Although the recurrence problem persists with ALL, it can be largely overcome in acute nonlymphocytic leukemia by marrow transplants when the patient is in the first complete remission (*89, 90*). The major remaining problems are the high incidence of fatal interstitial pneumonitis associated with cytomegalovirus and the high incidence of acute and/or chronic GVH disease, despite the MHC identity and MTX prophylaxis for the latter disease (*65, 91, 92*).

However, complete prevention of GVH disease may not be the optimal objective because, as in animal models (*30*),

GVH disease in man may exert an antitumor effect (*65, 93*). A statistical analysis of the causes of death of patients with acute leukemia who received transplants with allogeneic marrow revealed that they exhibited significant acute GVH disease and had a significantly lower rate of leukemia relapse than did allogeneic or syngeneic marrow recipients who exhibited no GVH disease (*94*). More recently, the occurrence of *chronic* GVH disease was also reported to be associated with a decreased incidence of leukemia relapse and longer survival (*95*). The results suggest that GVH disease induced by normal marrow has an antileukemic effect. The target antigens have not been identified. Various approaches, such as those investigated in animal models above, are being explored for the control and use of GVH disease in man.

Autologous marrow from patients in remission is being reinfused after supralethal chemoradiotherapy to treat hematologic and some nonhematologic neoplastic diseases in humans (*65*). The problems anticipated are their resistance to chemoradiotherapy and the contamination of the infused marrow by tumor cells. Scientists are now attempting to eliminate selectively tumor cells from infused marrow by using specific monoclonal antibodies (*96–98*).

The use of autologous lymphocytes as an adjunct to chemoradiotherapy has been proposed (*99*) but has not been tested adequately. This possibility deserves greater attention now that some human T-cell subsets have been identified (*100, 101*), cultured, and cloned (*102, 103*), and human cells have been made cytotoxic to tumor by sensitization with pooled normal alloantigens in vitro (*104*).

OVERVIEW OF STUDIES IN HUMANS

No valid or adequate adoptive immunotherapy studies on humans have yet been reported. The only approach which might represent it is that involving bone marrow transplantation after chemoradiotherapy, and the only evidence for an immunotherapeutic effect in that setting is the association between GVH disease and decreased leukemia relapse.

On the basis of studies in animal models, the prerequisite for adoptive immunotherapy in man are a minimal tumor load and a large number of viable effector lymphocytes immune to tumor and capable of persisting in the host for some time. These prerequisites would suggest that patients, free of tumor after surgery, chemotherapy, and/or radiation but whose tumors are most likely to recur, may be treated with chemotherapy or radiation, or both, in doses which would have an antitumor effect and would also be sufficiently immunosuppressive to delay rejection of immune infused lymphocytes (if allogeneic). The lymphocytes should be sensitized to tumor in vitro so that exposure of normal lymphocyte donors to tumor material is avoided.

Although the results of immunotherapy attempts in man are too sparse and cannot be rigorously evaluated for future directions by analogy to studies in animal models, it is essential that scientists continue clinical studies to: 1) document the existence of antigens on tumor cells that may be unique or shared with other tumors, other alloantigens, or differentiation antigens, and determine the optimal immunogens for in vitro sensitization to tumor; 2) identify the critical effect of cells generated and study their interactions with other cells and cell products; 3) maintain the effector cells in long-term culture, clone the cells, and test the interactions between cloned cells of different subsets; 4) control GVH disease; and 5) detect the antitumor reactivity of lymphocytes from patients bearing a tumor or who are in remission so as to determine ways to promote full expression of their antitumor reactivity by in vitro manipulations.

During the past decade, the assumptions that underlie the potential use of lymphocytes for tumor therapy have been largely validated in animals, animal models for therapy have been developed, some of the prerequisites for therapeutic efficacy in the models have been determined, and the problems those prerequisites pose for clinical extrapolation have been identified. Although no directly analogous approach has been effective in man, the reported antileukemic effect of allogeneic marrow in humans, as in animal models, is most encouraging. Several additional parallel developments in animals and man have markedly increased the likelihood that cell transfer will have a role in clinical cancer therapy. The existence of TAA has been demonstrated in many animal models. The clinical problem is far more complex but should more likely be solved now with the aid of monoclonal antibodies. Cytotoxic lymphocytes can be generated, maintained, and cloned with some demonstrated specific effector function in animal models, and the interactions of subsets of cells and the effector cell are being progressively better understood. Similarly, subsets of lymphocytes in man with different functions such as cytotoxicity, helper, and amplifier functions, are being identified by monoclonal antibodies, and human lymphocytes cytotoxic to tumor can also be generated and maintained. Moreover, the subsets can now be potentially manipulated, so that reactive T-cells can be eliminated and thereby prevent GVH disease, or suppressor cells eradicated for increased host reactivity to tumor and/or the immunotherapeutic efficacy of infused immune donor cells allowed. Continued exploration of these areas in animal models and, when appropriate, in man will undoubtedly better define the potential role of adoptive cell transfer as a specific component of cancer therapy.

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SECTION VIII

Tumor-Preventive Agents

INTRODUCTION

Chemoprevention refers to the use of chemical agents for the prophylaxis of the sequence of events that occurs following the exposure of an individual to a carcinogenic agent that results in the development of a neoplasm. With the growing realization that most human cancer is of environmental origin (*1*) and with an increasing understanding of the mechanisms of carcinogenesis, chemoprevention is becoming a realistic possibility. As discussed below, the process of carcinogenesis can be divided into five major sections: exposure, activation, fixation, progression, and expression. For an overall discussion of potential sites for intervention, *see* (*2*).

CARCINOGENIC MECHANISMS

Production of Initiated Cells

Prevention of exposure to carcinogens is clearly the most desirable way one can avoid developing cancers; however, advances in this area are principally a matter of education and complianee. Although the metabolic activation of carcinogens to proximal carcinogens (*3*) or the fixation of chemical lesions as biological lesions (*4*) can be averted in several ways, these preventive methods require intervention before or shortly after exposure to the carcinogen. Because exposure of most individuals to carcinogens is a cumulative lifetime event, such therapy would have to be of lifetime duration, the results of which would not be evident for several decades. These considerations impose severe constraints on the design, execution, and analysis of studies of this type in humans.

Progression of Initiated Cells to Cancer

In contrast to the problem outlined above, prevention of the progression and the expression of initiated cells (i.e., those cells bearing a latent carcinogenic lesion) presents many exciting possibilities for therapeutic intervention. Although little is known of the biochemical events that accompany the progression of an initiated cell to malignancy, evidence is increasing that drug-induced alterations of this process may be crucial to the overall incidence of human cancers. The process is accelerated by co-carcinogens called tumor promoters, compounds that are not themselves carcinogens but which potentiate the action of a carcinogen when administered after but not before that carcinogen. This delay in treatment with a promoter may be as long as 1 year in experimental situations, which shows that the initiator exerts a permanent effect on the cell. Whereas a single exposure to an initiator is required to produce this permanent change, repeated exposure to tumor promoters is required, and the effects of these exposures, though cumulative, are not permanent (*5*). Experimental systems in which promotion has been demonstrated include the skin, liver, colon, and urinary bladder. The most persuasive evidence that promotion is relevant to the clinical situation comes from research on tobacco carcinogensis. Research on constituents of smoke condensates has led to the conclusion that promoters constitute a major fraction of the biological activity when tested in mouse skin; furthermore, epidemiological evidence in man points to a major role for agents possessing the properties of tumor promoters (*6*). By accelerating the process of carcinogenesis, promoters cause the development of cancer within an experimental period or within the life-span of the individual. Thus in situations in which the initiating event is of insufficient magnitude to induce a neoplasm within the life-span of the individual, tumor promoters may be regarded as just as much a cause as the original carcinogen.

Natural and synthetic compounds related to and including vitamin A appear to have the property of preventing, and, in some instances, of reversing the process of tumor progression and appear to antagonize the actions of experimental promoters. These compounds will be referred to as retinoids (*7*). Interest in retinoids as a modality in cancer therapy began with the observation in 1925 that rats fed a vitamin A-deficient diet develop stomach carcinomas (*8*). Furthermore, experimental animals deficient in this nutrient developed hyperkeratosis of the skin and metaplastic changes in the epithelium of the respiratory, gastrointestinal, and urogenital tract (*9*). These changes were typical of changes observed early in the experimental induction of carcinoma in animals and correspond to precancerous lesions identified clinically. Direct relevance of retinoids to cancer came with the demonstration that the induction of chemically induced papillomas and carcinomas could be delayed or prevented by systemic treatment with natural retinoids (*10, 11*).

More recently, using defined in vivo models of organ-specific carcinogenesis, investigators have shown that retinoids can delay the development of chemically induced tumors, including carcinomas of the breast (*12*), bladder (*13*), and skin (*14*). The kinetics of inhibition point to the probability that retinoids are delaying the appearance of tumors rather than irreversibly preventing their development. For the most part, the experimental models require highly potent carcinogens administered at concentrations large enough to cause cancer in most of the exposed animals. This is unlike the situation in humans, in whom the highest incidence of cancer expected in a population of heavy cigarette smokers is 10% and after prolonged exposure to the carcinogenic stimulus. Retinoids may be more effective chemopreventive agents in this latter situation. For chemical structures of representative retinoids, *see* figure 8-1.

EPIDEMIOLOGICAL EVIDENCE FOR RETINOID ACTIVITY

That epidemiological studies of vitamin A in relation to cancer are far fewer than those based on experiments with animals is an accepted fact. Nevertheless, the results of the inquiries of epidemiologists are congruent with those of workers in chemical carcinogenesis.

The first glimmer of this came from the work of Bjelke (*15*), who conducted 2 retrospective case-control studies, 1 in Norway and 1 in Minnesota, and, in addition, prospective studies involving many thousands of individuals in both countries. Too little time has elapsed to allow one a definite look at the data from the prospective studies. However, the retrospective studies show a lower risk of

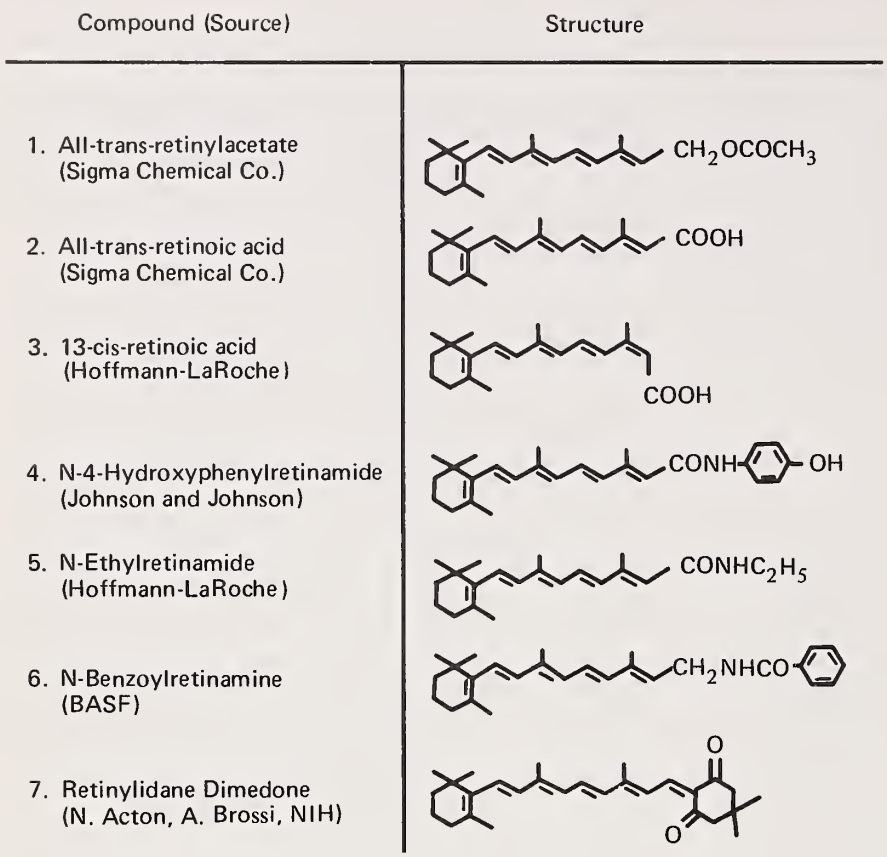

FIGURE 8-1.—Structures and commercial sources of natural forms of vitamin A (acetate and acid) and of several synthetic analogs showing high biological activity. BASF = Badische Anilin-und Soda-Fabrik of Ludwigshafen/Rhein, Federal Republic of Germany; Sigma Chemical Co. of St. Louis, Mo.; Hoffmann-LaRoche of Nutley, N.J.; and Johnson and Johnson of New Brunswick, N.J.

lung cancer associated with a high ingestion of foods containing vitamin A. Individuals classified as having a low intake of this vitamin had a fourfold to sixfold increased risk of lung cancer. MacLennan and co-workers (*16*) reported similar findings in a study of the Singapore Chinese.

Most recently, Mettlin et al. (*17*) studied the risk for several cancers associated with high, medium, and low levels of ingestion of dietary sources of vitamin A. They found a risk-reducing effect in dose–response associated with increasing levels of dietary vitamin A for cancer of the lung between light and heavy smokers; a twofold higher risk factor was determined among heavy smokers with a low intake. The same was found for cancer of the bladder and again within categories of smokers (*18*). Because heavy coffee drinking was previously associated with bladder cancer (*19*), the risk associated with varying levels of vitamin A was examined among those who ingested large and small amounts of coffee. Again, the risk-reducing effects of vitamin A were found in both categories of coffee drinkers. Preliminary analyses by the same authors suggest that a risk-reducing effect may be associated with increasing levels of dietary vitamin A for some other sites as well. Some segments of the U.S. population are chronically deficient in this nutrient and may be presumed to be at high risk (*20*). Others, by virtue of possible altered metabolism or tissue distribution, etc., may have inadequate retinoid concentrations at sites susceptible for environmental carcinogenesis. In support of this concept, a recent prospective study of about 16,000 British men found a significant negative association between initial plasma retinol levels and subsequent development of cancer. Those in the lowest quintile of retinol levels had an increased risk for cancer of 2.2-fold over those in the highest quintile. A greater than tenfold variation in mean serum retinol levels was recorded (*21*).

In 2 studies, however, such a correlation was not observed. Cohen et al. (*22*) assayed serum vitamin A levels in 67 patients with nonresectable lung cancer and found no decrease in serum concentrations among these patients compared with historical controls. Furthermore, they found no correlations between the estimated intake of this nutrient and serum levels. In a second case–control study, no strong protective influence for the regular consumption of vitamin preparations containing vitamin A was found in 800 newly diagnosed cancer patients (*23*).

With the experimental evidence reviewed below, there is a strong possibility that sections of the population are at increased risk because of low dietary intake of vitamin A and its precursors. Also, large variations in plasma retinol concentrations exist that negatively correlate with increased risk. The extent of these variations and the factors controlling them should be the subject of urgent study.

CHEMOPREVENTION STUDIES IN EXPERIMENTAL ANIMAL SYSTEMS

Effects of Vitamin A Deficiency In Vivo

Dietary deficiency in vitamin A intake is easily induced in experimental animals provided that time is allowed for

depletion of liver stores. Deficient rats have been demonstrated to be more susceptible to carcinogenesis of the respiratory tract (*24*), bladder (*25*), and colon (*26*). Newberne and Rogers induced marginal deficiency only, and excess vitamin A was more effective than standard concentrations in reversing the increased incidence observed in deficient animals.

Reversal and/or Prevention of Carcinogen-induced Tumors In Vivo

In practically all the studies reported here, retinoids were administered chronically in the diet after the subjects were exposed to carcinogens.

Urinary bladder.—The evidence is persuasive that carcinoma of the human bladder is due to urinary carcinogens. In experimental animals, bladder cancer can be readily induced by direct instillation of MNU (*27*) by ingestion of the bladder-specific carcinogens, i.e., OH-BBN (*28*) or FANFT (*29*). These carcinogens produce a spectrum of squamous and transitional cell carcinomas in rats and mice that histologically closely resemble the human situation. The synthetic retinoid, 13-*cis*-retinoic acid, reduced the number of cancers and severity of bladder lesions resulting from administration of OH-BBN to the rat (*13*) and mouse (*30*) or MNU to the rat (*31, 32*). Retinoid administration was effective when administered as late as 9 weeks after the carcinogen (*33*). Retinyl acetate, a natural form of vitamin A, strongly inhibited formation of squamous cell carcinomas and also transitional cell carcinomas at higher concentrations in FANFT- (*25, 34*) and OH-BBN-treated rats (*35*). In the latter study, retinyl acetate reduced the keratinization and squamous metaplasia associated with OH-BBN administration but did not induce histological changes in the absence of carcinogen.

Mammary gland.—As with the bladder, environmental factors are a major factor in the high incidence of breast cancer in this country, and premalignant changes can be detected long before invasive cancer develops. Excellent animal models, which closely resemble human cancer, are available with MNU (*36*). Synthetic retinoids delayed the appearance of tumors of the rat mammary gland induced by MNU and DMBA (*37*). The synthetic retinoid, 4-hydroxy-phenyl-retinamide, also had a pronounced antiproliferative effect on the rat mammary gland that did not appear to be due to inhibition of ovarian functions (*37*). Treated rats were capable of reproduction but milk production was depressed (Moon RC: Personal communication). Retinyl acetate at high dosage (323 mg/kg diet) inhibited MNU mammary carcinogenesis in the rat, but continuous administration to tumor-bearing animals did not influence tumor development. However, in tumor-free animals, continuous administration was required if tumor development was to be prevented (*38*). This study again underlines the reversible nature of tumor inhibition by retinoids. In a related study, Moon et al. (*39*) demonstrated that retinyl acetate would inhibit DMBA-induced mammary carcinogenesis in the rat and that the large doses required did not perturb liver function or estrous cycle. Spontaneous mammary carcinomas developing in C7H/AVY mice were inhibited by lifetime dietary supplementation with retinyl acetate at doses that caused severe skeletal abnormalities. However, spontaneous hepatoma incidence was decreased by about sevenfold at the highest dose level studied, i.e., 83 mg/kg diet (*40*). The synthetic retinoid, retinyl methyl ether, appears to be superior to retinyl acetate in delaying the appearance of DMBA-induced mammary tumors in the rat at dose levels causing no overt toxicity [(*12*); *see* fig. 8-2].

Skin.—Using the two-stage model of skin carcinogenesis in which cells are initiated by DMBA and promoted by twice weekly applications of croton oil, one can inhibit the formation of papillomas and carcinomas with topically applied retinoic acid (*41*). Skin carcinogenesis induced by DMBA in mouse strains differing in sensitivity to tumor induction was inhibited by the use of synthetic retinoid (Ro 10-9359), which interacted additively with inhibition by fluocinolone acetonide (*42*). The synthetic retinoid will also cause regression of established papillomas, an effect that cannot be attributed to changes in cell cycle parameters (*43*). Inhibition of DMBA-induced precancerous changes in rat skin, such as keratinization and cellular atypia, have been demonstrated with retinyl palmitate, the natural storage form of vitamin A (*44*). Recently, all *trans*-retinoic acid, 5,6-epoxyretinoic acid, and 5,6-dihydroretinoic acid, proposed active metabolites of retinoic acid, were equipotent in inhibiting both TPA promotion of DMBA tumorigenesis and the rise of ornithine decarboxylase consequent to TPA administration (*45*).

Recently, some have expressed concern over the apparent enhancing activity of retinoic acid in producing skin tumors (induced by UV light) in hairless mice. The initial study (*46*) which was done with toxic concentrations of

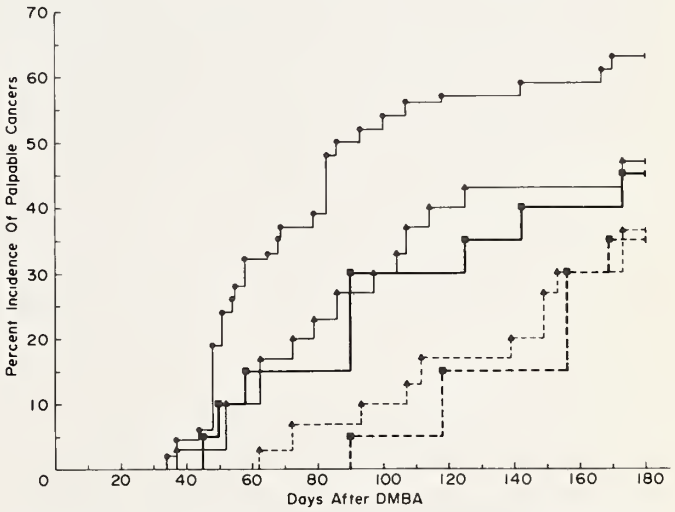

FIGURE 8-2.—Effect of retinyl methyl ether and retinyl acetate on the time of appearance of histologically confirmed palpable mammary cancers. Animals were placed on the various retinoid diets 1 wk after the intragastric instillation of 5 mg DMBA. Rats were palpated for mammary tumors twice weekly for the duration of the experiment. Diets fed (in micromoles of retinoid per kilogram of diet) were: ●, placebo; ■———■, retinyl methyl ether, 380; ■- - -■, retinyl methyl ether, 760; ▲———▲, retinyl acetate, 380; ▲- - -▲, retinyl acetate, 760. Figure is reproduced with permission of the publisher (*12*).

retinoic acid has been confirmed with the use of lower concentrations of retinoic acid that induced minimal skin erythema and epidermal hyperplasia (*47*). Because of these concerns, a bulletin was issued by the Food and Drug Administration warning of UV hazards during therapy for acne (*48*). Another study (*49*) has shown that retinoic acid cannot inhibit skin carcinogenesis induced by repeated applications of DMBA, and, in some situations, may actually enhance tumorigenesis. Thus retinoids appear to be potent inhibitors of skin carcinogenesis induced by a single treatment with initiator and multiple treatments with the promoter TPA. However, when multiple applications of the initiator are used for tumor induction, they can either accelerate carcinogenesis or have no inhibitory effect. This apparent promoting activity of retinoic acid must be viewed with concern (*47, 49*).

Colon.—An excellent model for human colon cancer is the DMH-treated rat. Vitamin A-deficient rats are more susceptible to DMH-induced colon carcinogenesis, but 13-*cis* retinoic acid markedly reduced (from 100 to 40%) its incidence (*50*). Additionally, vitamin A-deficient rats were susceptible to aflatoxin-induced colon cancer, a new site for this potent environmental carcinogen (*26, 50*). However, 2 studies failed to demonstrate a protective effect of retinoids on colon carcinogenesis. Neither 13-*cis* retinoic acid, given as 0.24 mg/g in the diet, or a trimethoxyphenyl derivative of retinoic acid influenced the number or severity of lesions induced by intrarectal administration of MNU (*51*). Similarly, Schmähl and Habs (*52*) observed no effect by Ro 10-9359 on DMH-induced colon carcinomas, nor, in contrast to many other publications, did they demonstrate an effect against OH-BBN-induced bladder carcinogenesis.

Stomach and small intestine.—Oral administration of DMBA to Syrian hamsters resulted in tumors of the esophagus, forestomach, and small intestine. Dietary feeding of high doses of retinyl palmitate profoundly decreased this incidence of tumors, but this early study was flawed by simultaneous application of both chemicals (*10*). In the hamster forestomach, squamous papillomas are reduced by high-dose retinyl acetate (*53*).

Respiratory tract.—In hamsters exposed to an intratracheal dose of benzo[*a*]pyrene–ferric oxide at a level designed to give an approximate 10% incidence of squamous carcinoma of the lung, the addition of small concentrations (3 mg/wk) of 13-*cis* retinoic acid chronically to the diet reduced the tumor incidence from 14/132 to 2/152; tripling the dose eliminated the development of carcinomas (*54*). In other studies, high doses of retinyl acetate elevated the incidence of benzo[*a*]pyrene–ferric oxide-induced benign tumors but the overall incidence appeared to depend heavily on the incidence of respiratory infections (*53*). In rats, the incidence of pulmonary carcinomas induced by intratracheal MCA is strongly dependent on vitamin A status. Enhanced susceptibility was demonstrated in rats fed a low-dose vitamin A diet prior to the onset of deficiency symptoms and while liver stores were high. The protective effects of an adequate intake (7.6 nmol/g diet) could not be further enhanced by an increase in the dosage of retinyl acetate or administration of all-*trans* retinoic acid (*55, 56*).

Tongue and buccal pouch.—In the hamsters, 13-*cis* retinoic acid (10 mg twice weekly) profoundly delayed the development of precancerous lesions and of tumors of the tongue produced by DMBA administration in the tongue on alternate days (*57*). A similar protocol by these authors demonstrated protection against DMBA induction of epidermal carcinomas of the buccal pouch (*58*). Conversely, high-dose retinyl palmitate applied topically to the hamster pouch reportedly enhanced DMBA carcinogenesis (*59*).

Liver.—All-*trans* retinoic acid supplied in the diet suppressed the development of hepatocellular carcinomas (that resulted when 3′-methyl-4-dimethyl-aminobenzene was fed to rats) from 38 tumors in 42 controls to 3 tumors in 27 treated animals (*60*). Spontaneous hepatomas developing in C3H/AVY mice were inhibited in a dose-responsive manner by retinyl acetate (*40*).

Summary of experimental animal studies.—In numerous studies conducted independently, retinoids have unequivocally inhibited carcinogen-induced carcinomas, papillomas, and, in some animals, hepatomas. In this respect, the positive reports more than outweighed the negative. Of concern to the further development of these drugs as chemopreventive agents are reports of the reversibility of the antitumor effect (*38*), which has been confirmed in vitro (*61*), and of the enhancement of UV- and DMBA-induced skin carcinogenesis under conditions of repeated carcinogen exposure (*46, 47, 49*). Although the former finding implies that chronic administration will be required for continued protection, the latter result is of more grave concern. Because this effect may be strictly dose dependent, enhancement too may be a consequence of increased cellular proliferation, which, in in vitro model systems, leads to more efficient fixation of carcinogenic damage (*62*). If, on the other hand, enhancement is a consequence of deeper biochemical mechanisms, as suggested by Schroeder and Black (*63*), then attention should be given to the possible tissue or cell specificities of these interactions.

Retinoid Effects on Organ Cultures

Prostate.—Laznitski and Goodman (*64*), using mouse prostate cultures in their pioneering work, have shown that the metaplastic and hyperplastic responses of these cultures to carcinogens such as MCA can be prevented by exposure of cultures to natural or synthetic retinoids. More importantly, if MCA is applied and the metaplastic changes allowed to develop, the addition of retinoids causes reversal of these changes. In the absence of retinoids, metaplasia persists and may progress. These studies have been confirmed and extended to *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine by Chopra and Wiekoff (*65*).

Trachea.—In the absence of retinoids, organ cultures of hamster trachea lose the characteristic columnar ciliated and mucous cells and enter a stage of squamous metaplasia. Addition of extremely small amounts of natural or synthetic retinoids causes reversion of the metaplastic state to normal (*66*). Hyperplasia and squamous metaplasia induced by carcinogenic forms of asbestos in the cultured

hamster trachea are prevented by coadministration of retinyl methyl ether (*67*).

Activity in Cell Cultures

Inhibition of neoplastic transformation.—Using the transformable mouse fibroblast cell line designated C3H/10T1/2 CL8 (10T1/2), Merriman and Bertram (*61*) determined that natural and synthetic retinoids are capable of delaying the development of neoplastically transformed cells that develop after exposure of these cultures to MCA. These authors treated cells with a single pulse of MCA, then, after 1 week, began continuous weekly exposure to retinyl acetate. By this technique, they demonstrated a straight-line dose-response relationship between retinyl acetate and inhibition of transformation. In this system, the ED_{50} and ED_{100} for inhibition were about 0.02 and 0.1 g/ml, respectively. These concentrations are below the normal (0.5 g/ml) plasma level in man. The effects of retinoids were reversible in that removal of retinyl acetate after 4 weeks resulted (after a further 5 wk in culture) in the development of the same number of transformed foci as in cultures never exposed to retinyl acetate. Thus, as in in vivo systems, the inhibitory effects of retinoids appear to be reversible and were produced without cellular toxicity. Transformation induced by X-rays has similarly been inhibited by synthetic retinoids (*68*).

Effects on established tumor cell lines.—Although in many systems concentrations of retinoids known to inhibit carcinogenesis in vivo or in vitro have had no such effects on established tumor lines growing in vivo (*69*) or in vitro (*61*), retinoids will cause growth inhibition and/or differentiation in other systems. At this time, it is unclear what relationship exists between chemopreventive activities of retinoids and their activities on established tumors. However, it is clear that the two properties are completely separable in a controlled in vitro system (*61*).

After the initial report of the growth inhibition and regression of transplanted rat chondrosarcomas by several retinoids (*70*) and growth inhibitory effects of orally administered vitamin A acetate or palmitate on transplanted adenocarcinoma (*71*) and Cloudman melanoma (*72*), studies in vitro have confirmed the ability of retinoic acid or retinyl acetate to inhibit tumor cell growth (*73*); murine melanomas were especially sensitive (*74*). Retinoic acid will also induce differentiation of a murine teratocarcinoma cell line (*75, 76*) and a human promyelocytic cell line (*77*). Lotan (*78*) has recently reviewed the activity of retinoids in normal and neoplastic eells.

STUDIES OF RETINOIDS IN HUMAN NEOPLASMS

Most of the published results of retinoid therapy in humans comes from Bollag's group at Hoffman-LaRoche in Switzerland (*79*). Patients with actinic keratoses and basal cell carcinomas were treated locally with retinoic acid and showed 40% (24 of 60) and 31% (5 of 16) complete remissions, respectively. Oral administration of retinoic acid has also been studied in patients with papillomas of the urinary bladder: Of 33 patients, 10 showed complete and 12 had partial remissions. Retinoid chemotherapy of established cancer is less efficient than conventional methodologies. Derivatives of retinoic acid have been tested in Germany for activity against oral leukoplakia, with positive results in 60% of 75 patients treated. In extended studies, about 45% showed partial or complete remissions. Although repeated therapy in recurring cases was usually effective, local and systemic toxicity was observed frequently (*80*). Use of 13-*cis* retinoic acid in the treatment of dermatological diseases resulted in a 40% incidence of blepharoconjunctivitis, the incidence of which was dose related and readily reversible upon drug withdrawal (*81*).

Chemoprevention of recurrent bladder cancer by 13-*cis*-retinoic acid was conducted by the National Bladder Cancer Cooperative Group A in a series of about 80 patients with a history of recurrent papillary superficial transitional cell carcinoma. Approximately 70% of these patients can be expected to develop further recurrences. The 13-*cis*-retinoic acid was administered to these patients at a level of 0.5–1.0 mg/kg per day orally for 6 months, followed by a 24-month observation period. Recurrence rates were compared with historical controls. This study was terminated early. Of the first group of 17 patients to complete 6 months therapy with retinoid, only 2 were free of disease at the end of the study period, a recurrence rate of 88%. In addition to the 17 patients completing the course, 7 were withdrawn because of unacceptable toxicity, for the most part identifiable as hypervitaminosis A. However, 1 patient showed acceleration of disease with extravesicular involvement. The apparent failure of investigators to demonstrate chemoprevention by retinoids in humans may be due more to the design of the trial than inactivity of the drug. Most experimental systems have shown retinoids to be ineffective against established tumors (*61, 69*), yet it is clear that, for recurrence to occur within 6 months in the patient population selected for study, many subclinical tumors must exist. If this argument is correct, then effective therapy must begin earlier, and it is in the identification and treatment of these high-risk groups that many problems are foreseen.

Other research currently being conducted by scientists at Hoffman-LaRoche includes an examination of the effects of 13-*cis*-retinoic acid on oral leukoplakia, cervical dysplasia, and actinic keratoses. No information on patient response is available at this time.

EFFECTS ON THE IMMUNE SYSTEM

In addition to inhibiting the progression of initiated cells to cancer, retinoids have also caused augmentation of immune responsiveness in experimental animals and in 1 clinical trial.

Experimental Animal Systems

Little to no adjuvant effect was noted for vitamin A when it was used alone or in combination with CPP in the treatment of the Swarm osteogenic sarcoma, L1210 leukemia, and DBRB mammary adenocarcinoma (*82*). However, in another combination treatment study, Meltzer and

Cohen (*83*) reported that tumor suppression mediated by BCG was enhanced by treatment of mice with the vitamin. A significant decrease in tumor incidence was observed with a transplantable, syngeneic, MCA-induced sarcoma when tumor cells were mixed with 6×10^4 CFU of BCG and injected intradermally into vitamin A-treated animals. Increased immune responsiveness to a given concentration of BCG organisms was noted in the treated mice. The enhanced host response to tumor by vitamin A is suggested by the reduction in the mean radiation dose required to control a transplantable murine fibrosarcoma (*84*).

The effect of vitamin A or its analogs on the cellular immune response was reported by several investigators. Floersheim and Bollag (*85*) and Jurin and Tannock (*86*) found that vitamin A treatment caused a significantly earlier rejection of skin grafts. Cohen and Cohen (*87*) demonstrated that the vitamin markedly increased the normal number of antibody-forming cells generated in the spleen in response to immunization with SRBC. The immunosuppressive effect of hydrocortisone on the response to SRBC was prevented by the simultaneous administration of vitamin A. Lotan and Dennert (*88*) demonstrated significant increases in cell-mediated cytotoxicity by 3 vitamin A analogs. This cytotoxicity, mediated by T-lymphocytes, was induced by ip injection of BALB/c S194 myeloma cells into C57BL/6 mice.

Chirigos (unpublished observations) examined retinyl palmitate and vitamin A in a tumor allograft system and found that treatment with the vitamin caused significantly earlier and more rapid rejection of tumor allografts. Retinyl palmitate treatment caused early rejection only at the highest dose (80 mg/kg) tested. The β-all-*trans* retinoic acid and the trimethylmethoxyphenol analog did not produce any enhancement in the SRBC footpad DTH assay nor result in an activation of macrophage nonspecific tumoricidal effect.

Vitamin A and other retinoids appear to have adjuvant properties in both humoral and cellular immune responses. In several transplantable syngeneic or hemisyngeneic tumor systems, however, the vitamin treatment alone had little to no effect on tumor growth. The adjuvant effect of the retinoids appears to be exerted through the cellular immune mechanism, probably by stimulation of the T-cell compartment.

Clinical Studies

Micksche et al. (*89*) reported that 13-*cis*-retinoic acid and vitamin A palmitate administered to 5 and 6 patients, respectively, with unresectable squamous cell carcinoma of the lung showed elevated immune responsiveness to recall antigens and an elevated in vitro blastogenic response of lymphocytes. Patients were treated with either 50 mg 13-*cis*-retinoic acid or 1.5 million IU of vitamin A palmitate twice daily for 3 weeks. All patients were described as exhibiting low toxicity that was seen as lamellar desquamation of the skin in every patient and cheilitis in 50% of them. Disease progression was slow during a 6-month treatment period, but no controls were included for comparison.

PROBLEMS ASSOCIATED WITH THERAPY

Lamellar Desquamation of Skin

Extremely high intake of natural or synthetic retinoids results in erythema of the skin and subsequent desquamation. Whereas part of this toxic reaction is probably due to intrinsic vitamin A activity that causes an excessive secretion of mucus by the epithelium, the vitamin has, in addition, a destabilizing action on membranes (associated with the terminal polar grouping on the side chain). Synthetic analogs have been developed in which this grouping is blocked and toxicity is lessened.

Liver Toxicity

Administration of natural esters (e.g., vitamin A palmitate or acetate) results in excessive hepatic deposition of long-chain storage esters and subsequent toxic symptoms. Administration of retinoic acid or 13-*cis*-retinoic acid overcomes this problem because conversion of storage esters cannot occur.

Inadequate Tissue Levels

Natural vitamin A esters are stored in the liver and released as retinol which binds to a high-affinity, plasma-binding protein. Administration of high concentrations of natural retinol esters results in liver storage but not in an elevation of plasma or nonhepatic tissue levels. Generally, synthetic retinoids are not bound to the plasma-binding protein and do not suffer from this problem, but occasionally they are localized in target tissues, e.g., breast with 4-hydroxyphenyl retinamide (*37*).

We hope that the development of new synthetic retinoids that are not stored in the liver nor bound to the plasma-binding protein have a higher therapeutic index as a consequence of decreased nonspecific toxicity and that they may exhibit an organ-specific concentration that will decrease many of the problems associated with the administration of natural retinoids (*90*). The NCI, through contract mechanisms, and several drug companies are actively involved in this search. For reviews of many synthesized compounds, *see* (*66, 91*).

Reversibility of Antitumor Action of Retinoids

In studies with cell cultures (*61*) and intact animals (*38*), the inhibition of chemically induced cancers depended on the continued presence of retinoid. Thus in clinical prophylactic studies, patients may be required to undergo lifetime therapy for continued protection.

CONCLUSIONS

Several conclusions can be reached:

1) In experimental animals and in vitro systems, retinoids retard the progression of cells to cancer and can reverse premalignant changes.

2) Epidemiological evidence in humans and in studies with vitamin A-deficient animals points to a correlation between lack of the vitamin and susceptibility to cancer.

3) In in vivo situations including man, high tissue levels of natural retinoids cannot be attained because of liver

storage (alcohol or esters of vitamin A) or nonspecific toxicity of the vitamin A acid. Synthetic analogs suggest a way to circumvent these problems.

4) In addition to effects on initiated cells, high dosage of vitamin A also enhanced immune responsiveness, probably by T-cell stimulation, which suggests that therapy of existing tumors may be feasible.

5) Therapy is thus possible for those patients who are tumor free, but at high risk of developing a primary or a recurrent neoplasm, and those with disease who can be expected to benefit from an augmented immune response.

RECOMMENDATIONS FOR FUTURE RESEARCH

Development of More Potent and Less Toxic Retinoids

Success in this direction could come from the synthesis of compounds of greater intrinsic activity, as was achieved with the steroids, and/or by more selective organ distribution, i.e., site targeting (*92*). Several investigators are working under NCI contracts in this area of research, and it seems that little further stimulus is required.

Design of New Clinical Trials for Chemoprevention

Several synthetic retinoids have demonstrated potency in experimental chemoprevention trials (e.g., 13-*cis*-retinoic acid, 4-hydroxyphenylretinamide, and retinyl methyl ether). The failure of the bladder cancer chemoprevention study can easily be attributed to poor design because most of the patients' latent tumors were too advanced for effective therapy. With this in mind, it is clear that chemoprevention trials must be conducted in apparently well patients, a restriction that may preclude many studies. Even in identified high-risk groups, the initiation of a trial may perturb the experimental results, and side effects may limit effectiveness. For instance, African albinos are a seemingly ideal population for study of chemoprevention because they almost uniformly develop squamous carcinoma of the skin which is fatal to most patients due to local invasion. The major etiologic factor is sunlight (UV-B irradiation in the 290- to 320-nm range). The prevalence of this disease suggests that adequate numbers of patients may be available for clinical study.

However, several obstacles preclude the immediate implementation of a chemoprevention study in this population:

1) Inasmuch as the major etiological factor is sunlight, the prevalence and natural history of this disease may be altered drastically through education and the provision of protective clothing and effective topical sunscreens. Implementation of these measures would seem to be of highest priority on humanitarian grounds, although avoidance may be impossible in control groups.

2) Because few data are available on the natural history of squamous cell carcinoma in the natural environment, they must be acquired and the impact of conventional preventive measures assessed. Effective protection from sun exposure may sufficiently alter the prevalence and progression of this disease so that it is no longer a useful model for the study of systemic chemoprevention.

3) Additional preclinical and clinical data are required so that selection of the most appropriate systemic chemopreventive agent is facilitated. The carcinogenic effect of UV-B irradiation can be abrogated in several ways. The first is light absorption by way of oral ingestion of a sunscreen. For example, carotenoids taken orally reach the stratum corneum and can absorb UV light. The carotenoids (C-40's) currently available for clinical trial absorb at about 400 nm and above, whereas the carcinogenic rays are in the 290- to 320-nm range. Preliminary data suggest that one or more carotenoids under development may absorb in the proper range, but data on their ability to prevent UV-B-induced carcinogenesis are fragmentary. The second approach is classical chemoprevention with retinoids. Retinoic acid has three side effects which warrant consideration prior to use in albinos. Firstly, it results in thinning of the stratum corneum. In albinos, hypertrophy of the stratum corneum is a mechanism of protection from UV because the protein is effective in absorbing these rays. Loss of this protective mechanism could actually facilitate carcinogenesis. Secondly, retinoic acid causes skin erythema which is exacerbated by sun exposure. Intolerance may be a treatment-limiting factor in albinos. Thirdly, animal studies have shown an enhancement of UV-induced skin carcinogenesis by retinoids (*47*). This disturbing finding needs further research before human trials can be initiated.

Immune Enhancement

Effects of high-dose retinoids on the immune system appear encouraging and should be actively pursued at the clinical level. Research in this area is not a nutritional fad but has a sound experimental background.

Priorities for Future Clinical Research

The potential clearly exists for the chemoprevention of human cancer; however, many problems arise. Currently available compounds have appreciable toxicity which may preclude testing in apparently well patients, and little is known of tissue distribution or organ specificity of the synthetic retinoids, which limits their rational use against specific tumors. At this stage of our knowledge, intervention carries with it the risk of augmentation of the disease especially when directed against UV-induced carcinogenesis. In view of the intriguing prospective and retrospective studies showing a negative correlation between vitamin A intake or plasma retinol levels and cancer incidence, it is recommended that such associations be further documented, and, if validated, that prospective intervention-type studies be conducted with natural forms of vitamin A. Correlations between intake, plasma levels, and tumor incidence could then be studied with little risk in large numbers of patients.

Basic research toward the development of more potent and/or organ specific retinoids should be pursued as described in the Program Operations Sections of this report.

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SECTION IX

Selective Updating Through Workshops Organized by the Subcommittee:

Immunoaugmentation Agents
Maturation Factors
Macrophage and Natural Killer Cells
Antibodies
Cytokines
T-Cells

INTRODUCTION

Six workshops were organized and sponsored by the BRM Subcommittee of the Board of Scientific Counselors of the Division of Cancer Treatment of the NCI in selected areas in which information was to be critically reviewed and future directions outlined and discussed in the light of that review. Each workshop was the responsibility of several Subcommittee members; the Workshop on Maturation Factors and Cancer was organized primarily through the invaluable efforts of Dr. Malcolm A. S. Moore, Sloan-Kettering Institute for Cancer Research, who acted as an ad hoc member of the Subcommittee on that occasion.

The proceedings of those workshops have been summarized by the chairmen of the organizing subcommittees, and these summaries are presented in this Section. The detailed proceedings are being published by Raven Press within the series, "Progress in Cancer Research and Therapy."

The titles of the workshops, the names of the chairmen of the responsible organizing subcommittee and the dates they were held are listed in table 9-1.

WORKSHOP ON IMMUNOAUGMENTATION AGENTS

Tumor immunotherapists entered the decade of the seventies with an enthusiasm and optimism which seemed soundly based on the early successes of Mathé in ALL, Klein in skin cancer, and Morton in melanoma. This enthusiasm propelled the field forward in directions which, in retrospect, seem at times to have been illogical. Moreover, methods were frequently imprecise or even inappropriate. Finally, the failure of immunotherapy to alter substantially the clinical course of human cancer led to some skepticism. Tumor immunotherapy enters the decade of the eighties as part of a new program of broader scope, i.e., biological response modification. There is hope that progress through this new decade will be made with greater precision and logic. This conference on augmenting agents was the first of a series sponsored by the Division of Cancer Treatment, NCI, and participants had as their objectives: 1) the identification of new agents for the BRM Program, 2) an assessment of their readiness for clinical development, and 3) an assignment of priorities for clinical development.

The first objective was accomplished. Detailed reports were provided on fractionation of BCG and *C. parvum,* as well as the current status of other natural products, such as *N. rubra* CWS, microbial polysaccharides, and *S. aureus* phage lysate. Equally detailed reports were presented on various IF inducers, synthetic polymers, and miscellaneous agents that could not be categorized otherwise on the basis of their source, structure, or mechanism of action.

The criteria for the second objective include such factors as the availability of a uniform product; toxicity data suggesting reasonable safety; evidence that a biological response of consequence in cancer control is modified; identification of a method for establishment of the optimum dose, schedule, and route of administration in man; and the demonstration of a significant antitumor effect in animal models.

Some of the inconsistent results with BCG immunotherapy trials that led many investigators to doubt its clinical utility can be traced to the multitude of preparations used. These BCG preparations varied widely in strain characteristics, the number of viable organisms per dose, and the quantity of debris. It is not surprising that the biological effects of these preparations also varied widely. The consistent availability of a uniform product is an essential prerequisite for the conduct of clinical studies.

Toxicity data are routinely gathered before the clinical application of any therapeutic agent. However, special consideration must be given to unusual problems which may be posed by these new categories of agents. For example, the long-term consequences of the chronic stimulation of the RES that may be produced by substances cleared by this network but not rapidly degraded and eliminated are now known. Will truly effective nonspecific stimulation of the immune system induce autoimmune disease?

When administered by the customary percutaneous routes, the first generation of augmenting agents (the microbial agents) generally failed to modify the biological responses under surveillance, although in the few instances when it was measured, they did so on iv administration. Improved application of first generation agents, availability of newer materials, and improved monitoring techniques have led to demonstrable modification of biological responses. The question that remains is whether these responses are important in the control of tumor growth. For example, Herberman presented a considerable quantity of data indicating that various BRM influence NK cell activity. What is the in vivo significance of this observation? With transplantable animal tumors, there is a correlation with survival for those tumors sensitive to NK cells in vitro. No data on primary autochthonous tumors in man and animals are available. Do alterations of macrophage number or function, or both, influence tumor growth? The data suggest that the answer is affirmative, but additional research is required. At present, we must focus on biological responses of only putative clinical significance.

In the early clinical trials of immune augmenting agents, dose, route, and schedule of administration were most often arbitrarily selected. Failure to demonstrate a therapeutic benefit and differences in results between trials were frequently attributed to variations in these parameters. The clear demonstration in animal models that immune modulation and control of tumor growth are both dose dependent makes careful phase I studies a necessity. How can optimum dose, schedule, and route be determined? Toxicity may not be dose limiting with many of these materials. Furthermore, the MTD may not be the optimum immune augmenting dose. Therefore, identification of optimum dose will likely be linked to the optimum modification of a biological response. Of necessity, this biological response must be one that is correlated with antitumor effect in animals and is also measurable in man.

An important criterion in the evaluation of a BRM is the demonstration of a reasonable antitumor effect in animal models. The appropriateness and utility of animal models have been matters of debate for over a decade. Bartlett

TABLE 9-1.—*Workshops sponsored by the BRM Subcommittee*[a]

Title	Chairman	Meeting dates
Immunoaugmenting Agents	E. Hersh	March 11–13, 1980
Maturation Factors and Cancer	M. A. S. Moore	June 12–14, 1980
Role of Macrophages, Natural Killer Cells, and Antibody-Dependent Cellular Cytotoxicity in Tumor Rejection and as Mediators of Biological Response Modifier Activity	M. A. Chirigos	December 10–12, 1980
Hybridomas in Cancer Diagnosis and Treatment	M. Mitchell	February 23–25, 1981
Lymphokines and Thymic Factors and Their Potential Utilization in Cancer Therapeutics	A. L. Goldstein	March 2–4, 1981
The Potential Role of T-Cell Subpopulations in Cancer Therapy	A. Fefer	June 1–3, 1981

[a] All the workshops were held in Bethesda, Maryland, except the one dealing with the lymphokines, which was held in Frederick, Maryland.

opened the conference by addressing the role of animal models in evaluation of the therapeutic potential of immunostimulants. He described the basic requirements of an animal model for cancer immunotherapy as an established tumor in a histocompatible host. Such models have existed for several years, and many of the agents discussed during this conference have demonstrated therapeutic efficacy in these models. Unfortunately, therapeutic efficacy is usually represented by some modest retardation of tumor growth or equally modest improvement in survival. Increases in cure rate are usually critically dependent on a small residual tumor burden, a situation difficult for clinicians to achieve in many clinical settings. Nonetheless, these models have generally reflected the outcome in man. Intralesional therapy in the guinea pig hepatoma model has been validated in human melanoma. The modest increment in survival produced by systemic administration of immune stimulants in animals is likewise reflected in a modest increment in survival when used similarly in man. The urgent need is for better therapies. Are those better therapies among the agents discussed during this meeting? Almost all have produced success in one or more animal models. For what measures of success are we searching? A benchmark can be established only when we have in hand an agent of meaningful systemic utility in man. The performance of such an agent in animal models can then serve as a standard for comparison of agents with similar mechanisms of action and perhaps other agents as well. For example, it seems that intralesional BCG in the guinea pig hepatoma model can serve as a benchmark for intralesional therapy of dermal disease in man. Although some are trying to extend the application of this approach in man (i.e., intralesional treatment of primary epithelial tumors prior to definitive conventional therapy), a need remains for effective systemic therapies, and a corresponding benchmark is not available. Operationally, selections for clinical development can be made by comparisons of different agents in the same animal model. Investigators have attempted such comparisons but generally only a single dose, route, and schedule of administration were used for each agent. This is an area in which the Division of Cancer Treatment has considerable expertise and can be most helpful in providing objective and thorough comparisons.

Because of these present limitations in our ability to assess clinical readiness quantitatively, the agent selection process will be partly empirical at the outset. As selected agents undergo systematic clinical development, the knowledge gained will enlighten our empiricism and make the selection process more rational.

With a general view toward these criteria, but with the focus on antitumor effect, what is the current state of preparedness for clinical development of the various materials discussed? The first day of the workshop was devoted to natural products and their subcomponents. This seems appropriate as these materials dominated clinical trials for the greater part of the seventies. The use of BCG or any other living microorganism has certain obvious disadvantages. Furthermore, it has been repeatedly speculated that with the use of fractions of the microbial adjuvants, therapeutic efficacy would be maintained or increased and toxicity would be reduced. Ribi described a portion of his extensive experience with BCG fractionation. Indeed, 300 μg BCG cell walls attached to oil and suspended in saline is effective intralesionally in the guinea pig hepatoma model as a 6×10^6 viable organism (31 of 53 vs. 30 of 51 cures, respectively). Moreover, fractionation approaches will likely allow the eventual separation of toxic and therapeutic effects, the dissection of mechanisms of action, and a broadening of routes of administration. However, no data indicated a therapeutic superiority over whole living BCG in an animal model. In man, Vosika demonstrated tumor regression with intralesional administration of CWS, *Mycobacterium smegmatis*/P3/oil with a series of 17 patients with melanoma. The magnitude and frequency of response seem similar to those reported by others with living BCG.

Tuttle described his experience with mechanical and chemical fractionation of *C. parvum*. Again, these procedures will likely allow separation of toxic from therapeutic effects and allow a dissection of the mechanism of action. The sensitivity of certain fraction functions to suppression by oxidation with sodium periodate and regeneration by reduction with sodium borohydrate is exciting. Pyridine extraction yields a fraction, 300 μg of which in combination with muramyl dipeptide and P3 is as effective as 10,000 μg whole cells. However, relative efficacy on a weight basis aside, the magnitude of the therapeutic effect of whole cells has not been improved upon significantly.

The therapeutic efficacy of equal amounts (300 μg) of *N. rubra* and BCG CWS was compared in various animal

models; on an equal-dose basis, *N. rubra* CWS seemed therapeutically superior. For example, it produced 6 of 13 cures in the spontaneous mammary adenocarcinoma model versus only 2 of 17 with BCG CWS. However, full dose–response curves are required for determination of a real difference between the 2 materials. A clinical trial is currently in progress in which patients with stages I through IV lung cancer are randomized after primary treatment to no further therapy or *N. rubra* CWS. At present, 60 patients are on the control arms with a median survival of 9 months, compared with 54 treated patients with a median survival of 15 months. Although this difference is significant ($P = 0.03$), its magnitude is modest and questions remain regarding the comparability of treatment and control groups. These factors, coupled with a lack of efficacy in the guinea pig hepatoma model, dampen enthusiasm for this material.

Bomford discussed several microbial polysaccharides and compared their activity with that of *C. parvum.* These materials are less potent activators of macrophages and have considerably less antitumor effect when used intralesionally. Attempts at chemical modification, such as oxidation, carboxymethylation, phosphorylation, and acylation have not yielded significant increments in therapeutic activity. It seems clear that additional basic research will be required before clinical application beyond that already in progress can be considered.

In patients with only dermal melanoma metastases, BCG is of clear therapeutic benefit. Its effect in the treatment of other tumors such as ovarian carcinoma, lymphoma, stage I non-oat cell carcinoma of the lung, and superficial bladder cancer may be validated by trials currently in progress. If a role for BCG in the conventional treatment of cancer can be substantiated, a less toxic substitute would have definite advantages. However, the current limited function of BCG in cancer therapy cannot alone justify an expansion of clinical research with microbial fractions. Fractionation studies of BCG and other microorganisms show promise of elucidating mechanisms of antitumor action and are deserving of continued support for this reason.

The phage lysate of *S. aureus* is a complex material containing metabolites of the bacterium, heat-stable and heat-labile antigens, solubilized products of the cell wall, and culture medium ingredients. This complex material has biological activity as manifested by its mitogenic capacity, ability to induce synthesis of IgG and IgM, and ability to stimulate IF. The antitumor potential of this material has not been extensively studied in animals and, as reported, human use has been limited to a single trial in patients with multiple sclerosis. Although *S. aureus* phage lysate has promising biological activities, data are insufficient at present to justify clinical trials in human cancer. Further fractionation and appropriate basic and preclinical studies are recommended.

An elegant series of experiments with endotoxin in 2 sensitive and 2 resistant animal tumor models was described by North. The systemic administration of endotoxins produces core necrosis of dermal tumor transplants in sensitive and resistant models. The remaining rim of healthy tumor regresses in sensitive models, whereas regrowth occurs in resistant ones. Complete tumor regression requires T-cells and produces a state of immunity which can be adoptively transferred by cells. The antitumor effect of endotoxin is most marked when given at the height of the host's immune reaction and is abrogated by the development of suppressor cells. The ability of endotoxin to induce core tumor necrosis after systemic administration would make most clinicians enthusiastic, particularly when coupled with the ability of this material to induce IF and produce a peripheral leukocytosis. This enthusiasm must be tempered by the knowledge that 1) complete regression of the necrotized tumor requires an immune response, and 2) the treatment is not nearly as effective for metastases in nondermal sites.

The second day of the workshop was devoted to IF inducers. The high cost and scarcity of IF led to a search for its inducers, a search that has been in progress for more than 10 years. The IF inducers have an additional advantage in that several types of IF are produced; this mixture may be more effective than a single type administered exogenously. In addition, the use of IF inducers may achieve higher titers, both systemically and locally at the sites of production, than could be achieved by the administration of exogenous IF. It was soon appreciated that double-stranded nucleic acids induced high levels of IF, and poly I:poly C was developed and brought to clinical trial. This material not only induced IF but also enhanced immunity and had a direct antitumor effect. Although toxicity was modest even up to 12 mg/kg per dose, antitumor effects were also modest. This lack of antitumor activity was believed due to the short $T_{\frac{1}{2}}$ of poly I:poly C that resulted from a plasmalytic activity. A polycationic shield was built around poly I:poly C to prevent endonucleolytic attack, which in turn "unzips" the molecule. Thus poly ICLC was developed. Poly ICLC is more stable and retains the ability to induce IF; unfortunately, it is more toxic than poly I:poly C. Levine at the NCI and Krown at Memorial Sloan-Kettering Hospital have taken this material to clinical trial. The MTD, related in part to age and other clinical characteristics, varies from as little as 1 to as much as 12 mg/m^2. However, even in adults with similar clinical profiles, the MTD varies widely and is unpredictable. Furthermore, a wide range of IF was produced for any given dose. However, in both studies, doses of 8 mg/m^2 seemed to induce IF consistently at levels that ranged up to 2,000 μ/ml. An optimal schedule has not been determined, and this lack is complicated by the phenomenon of hyporesponsiveness. Krown overcame this hyporesponsiveness partly in the Memorial Hospital study by progressively escalating the dose. One complete remission in a patient with ALL was noted during the phase I trial at the NCI. Several phase II trials are in progress, each with a different dose and schedule of administration. These seem to have been determined by empiricism enlightened by a prior phase I experience and further modified by current experience. One patient with AML sustained a partial remission, and 1 or 2 patients with multiple myeloma also seemed to be responding. This pattern of response is similar to that previously demonstrated for exogenous IF. These responses are not without side effects. Almost all patients become febrile, 75% have hematologic

toxicity, 50% develop nausea, and 25% become hypotensive.

Investigators' attempts to reduce the toxicity of poly I: poly C while maintaining IF-inducing properties include enhancing endonuclease degradation by the mismatching of bases. This procedure does reduce toxicity, but a question remains as to whether this readily hydrolyzable material will 1) actually induce IF in man and 2) show antitumor activity.

Morahan discussed synthetic polyanions including polycarboxylates, polysulfates, and polyphosphates. These materials affect various biological functions, in addition to their ability to induce IF, that include antimicrobial and direct antitumor activity, activation of macrophages, effect on NK cells, and adjuvanticity for antibody formation. Induction of IF by polyanions has been demonstrated in the mouse and man but not in swine, rabbits, rats, or guinea pigs. Serum IF concentrations are low and reach only 1–200 μ/ml; also, hyporeactivity occurs. The antitumor and antiviral effects of polyanions do not correlate directly with IF production. For example, the antitumor effect of pyran is only partially abrogated by anti-IF serum.

Blue Tongue virus is a retrovirus which is pathogenic in ruminants but not in man; its vaccine strain BT-8 induces 680,000 μ/ml IF in mouse serum. By comparison, poly I: poly C yields 17,000 μ/ml. Plasma IF levels greater than 100,000 μ/ml have also been induced in rabbits and monkeys; IF levels remain high for about 7 days after a single treatment. In vivo, UV-irradiated, noninfectious Blue Tongue virus is about 10% as effective as the nonirradiated vaccine. High yields of IF have been induced in vitro with rabbit, hamster, chicken, dog, cat, and pig cells, as well as human leukocytes and human bladder cancer cells. In human leukocytes, results with this virus are similar to those achieved with the Cantell strain of Sendai virus. Blue Tongue virus inhibits the growth of B16 cells in vitro, but data on the growth of B16 in vivo are fragmentary. Jameson suggests that this virus vaccine may be a more stable and less toxic IF inducer than is poly I: poly C or poly ICLC. Data generated with human tumor cell lines suggest that repeat induction is possible. The problem with Blue Tongue virus is that in vivo, its ability to induce IF declines drastically as the host develops immunity to it. The hope is that an active, nonimmunogenic component can be identified.

Bru-Pell, an aqueous ether extract by Brucella strain 456 (Rough), is an IF inducer and macrophage activator. The IF induction is dose dependent in mice with maximum plasma titers of 2,000 μ/ml at 6.5 hours. Ability to induce IF in other species was not reported, nor were data presented on hyporesponsiveness. A single ip injection of Bru-Pell will protect mice from S180 ascites formation when given on a schedule varying from day −4 to day +7, with equal protection throughout. Immunogenicity of an L1210 vaccine was enhanced in a prophylaxis experiment, and the number of pulmonary metastases in a Lewis lung carcinoma model was reduced. Bru-Pell was also effective in the LSTRA chemoimmunotherapy model. Although no one reported efforts to correlate its antitumor effect with IF induction, Bru-Pell was said to be a better IF inducer than is BCG or *C. parvum*. The claimed advantage is the lack of depot accumulation. Further investigation of this product is indicated.

Stringfellow is investigating the antiviral activity, IF inducing capacity, and immune modulating effects of the pyrimidinoles; he presented data on the following 3 agents: 2-amino-5-bromo-6-methylpyrimodinole, 2-amino-5-bromo-6-phenylpyrimidinole, and 2-amino-5-iodo-6-phenylpyrimidinole. Some of these compounds are vigorous IF inducers in various species, e.g., IF has been induced in monkeys and in a human tissue culture system. An encouraging observation is the suggestion that prostaglandins may help overcome hyporesponsiveness. Studies on antitumor activity in animal models are preliminary. In the B16 melanoma system, the number of lung metastases is reduced, but almost all animals have some lung metastases and eventually die from them.

The available data on a whole spectrum of low molecular weight IF inducers was reviewed by McIntyre. Several of these, such as kanamycin and quinacrine, are used clinically for other purposes, but the data available on these substances were fragmentary. In most cases toxicity was high. Data on IF levels were often not available, nor was an antitumor effect demonstrated.

In summary, the major obstacles to the clinical application of IF inducers relate to their toxicity and the phenomenon of hyporesponsiveness. We can probably overcome the latter problem by alternating inducers, changing the schedule design, or targeting inducers to different cell populations. Additional preclinical research is required in this area.

Type II (immune) IF was discussed by Dianzani. Inducers of immune IF include: bacterial antigens (tuberculin) and toxins (*S.* enterotoxin A), lectins (PHA), enzymes (galactose oxidase), and oxidizing agents (periodate). Immune IF potentiates the antiviral activity of Type I IF elevenfold. Data for animal tumor models are limited. When P388 and immune or Type I IF, or both, are given ip daily for 14 days (starting on day 9), combination therapy produces a synergistic suppression of tumor growth. Type II IF has been partially purified, but its mechanism of action seems to differ from that of Type I because no cross resistance is seen. It seems unlikely that the above cited or similar inducers of Type II IF could be placed into clinical trial because of toxicity. At present, the leaders of the BRM Program contemplate clinical development of the Type II when adequate supplies of an appropriate material become available.

The third day of the conference was devoted to cancer therapy with synthetic polymers and other synthetic compounds. The session was opened by Makowka who discussed NED-137, a polymer developed by Monsanto Chemical Company (St. Louis, Mo.), the structure of which was not revealed. The material has a mean mol wt of 800 (range 400–1,200), is nontoxic, and is administrable ip, iv, and orally. The only animal tumor model in which it has been systematically evaluated to date is a transplantable MCA-induced bladder cancer in Fischer rats. This is a rapidly growing tumor; a subcutaneous implant of 0.5 cm diameter reaches several centimeters in size in a few days. If the tumor is not treated, the median survival is 2 weeks. When animals are treated with a single 30-mg/kg dose of

NED-137, median survival is increased to 6 weeks. Most studies are conducted in a model in which the tumor is excised on day 7. If no further treatment is administered, the tumor recurs locally and lung metastases develop. Untreated, all animals are dead by 5 weeks. A single treatment on day 0 produces 100% survival at 60 days with a subsequent drop-off. Treatment every 6 weeks yields 100% survival at 24 weeks. Effeetive over a wide range of doses (0.5–30 mg/kg), NED-137 was shown to be superior to various other augmenting agents, such as BCG, *C. parvum,* and pyran, in comparative studies.

This material is currently under study in other animal tumor models, and the effects on the immune system are under investigation but to date have been minimal. The increase in antibody-forming cells in the Jerne plaque assay is threefold to fivefold. Also, no direct cytotoxic effect on tumor cells was observed.

Falk used NED-137 clinically at a dose of 30 mg/kg every 6 weeks ip, iv, and orally in an initial group of 45 patients as part of a phase I study. No serious toxieity was noted. Studies with NED-137 given iv were then expanded to 120 patients with gastrointestinal cancer of all stages. Again little toxicity was noted. Therapeutic benefit was suggested by an attempted comparison of outcome with that of a historical control group. Meaningful conclusions were not possible because of the heterogeneity of the 2 groups and marked imbalance in important clinical parameters.

Pyran is a divinyl ether-maleic anhydride cyclic copolymer. The unfractionated mixture was subjected to phase I clinical trials a decade ago by Regelson and coworkers, but further clinical usage was abandoned because of serious toxicity. The material has not been fractionated and MVE-2, a low molecular weight fraetion, retains antitumor and biological response modification capabilities with diminished toxicity. Pyran is a potent modifier of biological responses; it induces IF, impairs coagulation, increases antibody production in the Jerne plaque assay, activates macrophages, decreases mitogen (PHA, LPS), induces lymphocyte blastogenesis, and protects against malaria in rodents.

Studied in a wide variety of animal models and immunoprophylaxis investigations, pyran was active in retarding tumor growth. Antitumor activity was also demonstrated in the adjuvant setting. The best results are achieved when the primary treatment is most effective. For example, LSTRA is sensitive to BCNU. When pyran is given prior to substantial recovery to tumor volume, cure rates approach 100%. If treatment is delayed, its effect is reduced. Alternatively, the Lewis lung tumor is resistant to Me-CCNU but only modestly so to pyran. These data indicate that the activity of pyran is critically dependent on tumor load. Extrapolation of these models to the clinic setting is fraught with difficulty because clinical staging techniques do not allow accurate measurement of the tumor load. In tumor-bearing animals, pyran alone has produced modest increases in survival and rarely some tumor regression.

Schlumberger discussed the antitumor effect of nonionic synthetic copolymer Bay i 7433 that was studied in various animal tumor systems; a dose-dependent effect was demonstrated. This material increased chemotaxis, but extensive investigations have not shown any other measurable immunologic effect. Rapid iv infusion produced a toxicity related to the viscosity of the polymer solution. Otherwise, this agent is almost completely without side effects.

A lipoidal amine, CP 46,665 activates macrophages and has some direct cytotoxic effect but is not an IF inducer. Administered iv (with Intra lipid), it localizes in the RES and has the ability to reduce lung metastases following surgical excision of a 21-day-old B16 melanoma transplant. This particular lipoidal amine has no effect in the L1210 and P388 models.

In summary, NED-137, MVE-2, Bay i 7433, and lipoidal amine have all demonstrated antitumor effect in relevant animal models. The performance of NED-137 in the rapidly growing, transplantable rat bladder carcinoma model is impressive. Ease of manufacture of a reproducible product, lack of toxicity in animals and man, and ease of administration are all important assets. However, the therapeutic efficacy of this compound requires further assessment in additional, more conventional animal tumor models. The antitumor effects of the lipoidal amine CP 46,665 have been mixed. That Bay i 7433 demonstrated antitumor effects in a wider variety of animal tumor models when treatment was initiated on days -6 to $+2$ suggests a critical dependence on tumor load.

Pyran has been tested most widely in animal models. A multitude of biological responses are modified, and fractionation markedly reduced toxicity. Questions remain regarding the preparedness of pyran (MVE-2) for clinical development. However, the data base for this compound is large by comparison. For this reason, MVE-2 has been empirically selected for clinical development. The best approach to assessment of the relative antitumor effects of the other 3 compounds is through direct comparisons in the same animal models. This approach plus the experience gained during the clinical development of MVE-2 should allow more rational decisions in the future.

The final day of the conference was devoted to miscellaneous substances and began with a discussion of possible immune modulation through regulation of prostaglandin synthesis. Prostaglandins are universal local feedback inhibitors. If the synthesis of prostaglandin is stopped, the response to various normal stimuli is increased. In the early 1970's, it was noted that the addition of prostaglandin to certain in vitro assays resulted in depressed T-cell proliferation and cytotoxicity, NK activity, ADCC, and macrophage function. Prostaglandins are produced by several types of cells, including macrophages and some tumor cells. The addition of prostaglandin synthetase inhibitors, such as indomethacin or aspirin, to in vitro immunology assays produces a mild stimulation of some functions.

Inhibitors of these naturally occurring fatty acids can be useful in cancer therapy in four ways: 1) As a mild immunostimulant, indomethacin increases DTH in patients and guinea pigs with combined immunodeficiency disease. 2) They restrict the increased macrophage prostaglandin production induced by some tumors, such as murine fibrosarcomas. 3) They hamper production of these hydroxy fatty acids by tumors, such as human breast and

renal adenocarcinomas, and indomethacin treatment of prostaglandin-producing animal tumors results in reduced tumor growth. 4) Macrophage production of prostaglandin induced by immunomodulators (e.g., BCG or *C. parvum*) is restricted. Induction of splenic suppressor cells by BCG or *C. parvum* may be through a prostaglandin mechanism.

The H2 receptor antagonists cimetidine and methiamide also affect the immune response. When added in vitro, low-dose cimetidine can increase the antibody response to SRBC twofold to threefold. High-dose cimetidine is directly cytotoxic. When used in vivo, both agents can increase the antibody response to SRBC by spleen cells from tumor-bearing mice. They are thought to act by depleting the H2 receptor-bearing suppressor T-cells.

A low-molecular weight material of low acute and chronic toxicity, DTC is only effective when used in vivo. It is active immunologically over a wide range of doses and is thought to induce production of a hormone specifically active in T-cell precursors. The addition of serum from DTC-treated mice to human E-cells in vitro generates HTLA+ cells (i.e., mature T-cells). Whereas DTC has no effect on B-cells, it increases T-cell-mediated responses, such as the primary IgG response to SRBC and PHA-and Con A-induced blast transformation; listerocidal activity of macrophages; ADCC to chicken erythrocytes; and delayed sensitivity to SRBC or BCG. It impairs B-cell-associated responses, such as PWM-induced lymphocyte transformation and circulating IgM levels. Alone, DTC has no mitogenic activity. No data have been accumulated on its antitumor effect, but these should be sought.

Munder described the use of allyllyso-phospholipids in cancer therapy. These compounds, which demonstrate variable antitumor activity, are most effective against Ehrlich ascites tumor and less active against the Meth A sarcoma. The mechanisms of action are unknown but may relate to macrophage activation. A direct antitumor effect in vitro against the Meth A sarcoma is suggested.

Tuftsin, a small peptide (threonine-lysine-proline-arginine) which activates macrophages and granulocytes, increases phagocytosis, motility, macrophage killing of bacteria after ingestion, and RES clearance of *E. coli* and *S. aureus*. The antitumor activity of tuftsin has not been systematically evaluated, but in the L1210 system, it increases survival from 12 to 15 days.

Amphotericin increases cell permeability, which enhances the uptake of some drugs, such as dactinomycin and BCNU. In the AKR leukemia model, BCNU plus amphotericin B yields 30–40% long-term survivors compared with none of either agent alone. Immunologically, amphotericin can serve as an adjuvant in humoral and cellular responses. Clinical trials are currently in progress and are designed for the study of drug resistance.

Hadden discussed 2 BRM: Isoprinosine, in worldwide use as an antiviral agent with negligible toxicity, increases lymphocyte proliferation in vitro to PHA, Con A, lymphocytes, and microbial antigens. Isoprinosine is as effective as thymosin in stimulating T-cells and as endotoxin in stimulating B-cells, and also activates NK cells. When used as an adjuvant in an L1210 vaccine prophylaxis study, it increased mean survival time. Some antitumor effect was noted in the S180 system in combination with IF. A new agent, NPT-15392, also increases lymphocyte proliferation to PHA, Con A, and PWM. Active E-rosetting cells are increased and T-cell markers are induced.

Azimexone (BM 12.531) is a 2-cyan-substituted aziridine with low toxicity (after parenteral oral administration) and no direct cytostatic activity. It had antitumor activity in various mouse and rat models. Data were presented on the activity of azimexone in the Lewis lung carcinoma, AKR leukemia, and a mouse fibrosarcoma. In the AKR leukemia, azimexone was equally effective against immunogenic and nonimmunogenic clones. A clear bell-shaped dose-response curve was demonstrated in all 3 systems, and the most effective dose was 25 mg/kg per injection. Antitumor effect also depended on the number of treatments given, with 6 generally preferable to 4 or 8. Most dramatic was the excellent correlation of antitumor effect with an increase in cytotoxic "autoreactive" cells. Mixing experiments demonstrated the presence of T-suppressor cells at high doses which correlated with a lack of antitumor effect. Preliminary phase II data are available. Oral doses of 900 mg/patient seven times produced a decrease in erythrocytes and a rise in leukocytes that were reversible in 14 days. A dose of 300 mg produced no change in clinicochemical parameters. Unfortunately, cytotoxic autoreactive cell activity was not evaluated.

A thiazolobenzimidazole, Wy-18,251, has the capacity to increase 1) levels of circulating T-lymphocytes, 2) delayed hypersensitivity to DNCB in CPP-suppressed guinea pigs, and 3) lymphocyte proliferation to suboptimal doses of Con A. The antimetastatic activity of this compound was investigated in the Lewis lung model and was schedule dependent. Effective daily treatment required high doses (150 mg/kg per day), whereas weekly treatment was effective at 1 and 5 mg/kg.

The data available on prostaglandin antagonists (indomethacin, aspirin), H2 antagonists (cimetidine, methiamide), DTC, tuftsin, Isoprinosine, and NPT-15392 relate largely to their biological responses, and the belief that they are important for the control of tumor growth is still fragmentary. The allyllyso-phospholipids have a variable antitumor effect. The suggested role for Wy-18,251 is as an antimetastasis agent, but the property has been studied in only 1 animal model to date.

The data on azimexone are encouraging, although the extent of antitumor effect needs further exploration. Have models been tested in which there is no antitumor effect? In the positive AKR model, does a modest increase in grafted tumor cells negate the antitumor effect? What are autoreactive cytotoxic cells? Are these NK cells? If they are NK cells, are the tumors responsive in vivo only those that are sensitive to NK cells in vitro? Can this parameter be measured in man? Despite these questions, azimexone seems most ready for clinical development. The observation that antitumor effect varies directly with the presence of cytotoxic autoreactive cells may provide an in vitro monitor for phase I studies in man.

In the preceding paragraphs, we made an effort to identify those agents most ready for clinical development. Our third and final objective was an assignment of priorities for clinical development. Because of our current inability to assess clinical readiness quantitatively, an

assignment of priorities must be largely empirical. Nonetheless, this is an essential step if the BRM Program is to move forward in an orderly fashion.

If a role for microbial agents in the conventional treatment of cancer can be substantiated, a less toxic, consistently uniform, readily quantifiable substitute would offer definite advantages. However, the current limited role of microbial agents cannot alone justify an expansion of clinical research with microbial fractions, and preclinical studies to date have not demonstrated a clear therapeutic advantage for fractions over whole organisms. As cited above, IF inducers offer several advantages over exogenous IF. The major obstacles to wider clinical application relate to toxicity and the phenomenon of hyporesponsiveness. Additional preclinical research is indicated before expansion of clinical application. The prospect that Type II (immune) IF can act synergistically with the Type I in suppressing tumor growth is exciting in view of the encouraging early results with Type I alone in various human neoplasms. At present, the BRM Program contemplates clinical development of Type II IF when adequate supplies of an appropriate material become available. A wide variety of synthetic materials have demonstrated antitumor activity and significant biological response modifying capability in clinically relevant animal models. Pyran (MVE-2) has been most widely tested in animals and for this reason has been empirically selected for clinical development. Azimexone has also been widely tested in animal tumor models and yielded exciting results in that a clearly well-defined bell-shaped dose–response curve exists, and antitumor activity is directly correlated with an increase in cytotoxic autoreactive cells. This material also may be ready for clinical development. Those materials which to date have only been studied for biological response modification capability (e.g., tuftsin) will require further preclinical testing in appropriate animal tumor models. Selecting agents for clinical development from among those that have demonstrated antitumor effects in relevant animal models will rely heavily on direct, objective, and systematic comparisons in the same or similar models. We hope these data, combined with the experience from the clinical development of MVE-2, will lead to a more objective selection process.

WORKSHOP ON MATURATION FACTORS AND CANCER

More than 30 polypeptide growth factors are reported in the literature (excluding factors influencing hematopoiesis and the immune system). Of these, NGF is probably the most fully characterized, and a reasonable amount of information is also available about EGF. However, for other factors, e.g., SGF, fibroblast growth, mesenchymal growth, glial, glial maturation, and all the rest, information is incomplete. The chemistry of most of these is unknown, their overall actions are unclear, and a better definition of their fundamental biological significance in vivo is necessary.

Inasmuch as information about NGF is fairly extensive, a discussion of it as a prototype of the growth and maturation factors may be useful. It is a small basic protein (mol wt, 13,500) that exists as a noncovalently linked dimer with a mol wt of 27,000. The linear sequence β subunit has been worked out; it bears a resemblance to the linear sequence of proinsulin.

The most persuasive argument for the biological significance of NGF has been obtained with an antibody prepared against it. This antibody, when injected into young animals, deprived them of their sympathetic nervous system, a procedure now known as "immunosympathectomy." Information on this autoimmune model was presented by Johnson and his colleagues. Their data provide the first in vivo evidence that sensory neurons of neural crest origin go through a phase of NGF dependence. The autoimmune approach provides important information regarding the role of NGF on the life cycle of the sympathetic and sensory neuron. In the future, it offers the potential for allowing us to examine the role of NGF in various cell types in utero and for determining the time of NGF dependence, the embryonic origin of neurons in cranial ganglia, and the secondary effects of destruction of most of the peripheral sensory system. The general autoimmune approach may be applied to other proteins, many of which have been classified as growth factors largely on the basis of their ability to affect cell growth or multiplication in vitro. The autoimmune approach offers a possible method by which a developing organism in vivo can be deprived of a putative growth factor. The applicability of the approach to other polypeptide factors would, of course, depend on the antigenicity of the peptide, the accessibility of the antibody to its synthetic sources and storage sites before and during the developmental event of interest, and the cross-reactivity of the antibody for the particular species under study.

The relevance of NGF to tumors of the nervous system was discussed by Guroff and by Goodman and Herschman. Among the neuronal tumors derived from neuroectoderm that exhibit differentiated function are the neuroblastomas, retinoblastomas, medulloblastomas, and pheochromocytomas. Inhibition of growth with induction of differentiated function can be seen in many neuronal tumors in the presence of NGF. Human melanomas also have large numbers of NGF receptors but exhibit no change in growth rate or alteration in morphology in the presence of NGF. The rat pheochromocytoma model, PC12, was cited as an important model of NGF action on tumor cells. The cell line grows as a rounded unremarkable cell, which, upon addition of nanogram amounts of NGF, exhibits a rapid and profuse outgrowth of neurites; the cells stop dividing and differentiate into sympathetic neurons. Receptors for a second growth regulatory peptide, EGF, were also present on the tumor cells, but EGF treatment caused neither neurite outgrowth nor hypertrophy and produced a modest increase in cell proliferation. When both NGF and EGF are added to the tumor targets at the same time, the cells undergo differentiation and cease dividing. In other words, the action of NGF appears to predominate and overrides the modest effects of EGF on cell proliferation. This mechanism appears to be due to the capacity of NGF to limit the synthesis of receptors for EGF. This important concept may provide a common mechanism by which

maturation factors act, i.e., by inhibiting synthesis of receptors for growth stimulatory (mitogenic) factors.

The session on EGF and fibroblast growth factors was introduced by Carpenter who emphasized that the complete amino acid sequence of EGF was known. As with NGF, excellent evidence is available for an in vivo role for this molecule. Enhanced proliferation of epidermal tissue can be seen in sites as diverse as the cornea, lung, trachea, and esophagus. It also inhibits gastric acid secretion, an observation attributed to a significant sequence homology with a small polypeptide (urogastrone), which also inhibits gastric acid secretion and possesses some EGF activity. This is yet another example of regulatory polypeptides possessing two quite distinct biological activities and extensive structural homology. Interaction between EGF and their receptors on appropriate target cells is a "down regulation phenomenon" with internalization and degradation of the hormone. The initial mitogenic effects of EGF probably do not require this internalization, but it is possible that DNA synthesis does. Isolation of membranes from the human epidermoid carcinoma (A-431), which has an exaggerated concentration of EGF receptors, allowed the purification of these receptors. Highly purified material was capable of acting as an endogenous substrate for basal and growth factor-stimulated protein phosphorylation. These data suggest an intimate association between the EGF receptor and a membrane protein kinase; they share several characteristics and the *src* kinase of sarcoma virus-transformed cells.

Herschman and Sorrentino addressed the question of pathways of growth control of 3T3 cells that can be stimulated to enter the proliferating pool by molecules as distinct as EGF and TPA. By isolating variants of 3T3 cells which are unable to mount a mitogenic response to specific mitogens, we can determine whether mitogens of diverse structure stimulate the same set or different sets of causal events distal to receptor occupancy. Are there common or distinct pathways to proliferation for different mitogens? Isolation of cell variants altered in receptor synthesis, ligand binding, or ligand-induced internalization can be enhanced by selection with a toxic conjugate between EGF and the A-chain of the toxic glycoprotein ricin.

The properties of PDGF, a heat-stable cationic protein with a mol wt of 35,000 released from the alpha granules of platelets were described by Scher. The PDGF component of serum is a limiting factor for the growth of fibroblasts in culture. This factor does not have to be present continuously to initiate fibroblast entry into the S phase. A brief treatment with PDGF makes cells competent to synthesize DNA and primes the cells to respond to other growth factors present in plasma. These latter factors, responsible for progression of the cells through S phase, are identified as somatomedins, a group of polypeptide hormones with insulin-like activity and structural homology to proinsulin. They appear to be synthesized by the liver in response to pituitary growth hormone.

Examination of the cellular growth response to tumor viruses and chemical promoters in the context of competence and progression may permit functional analogies between these agents and specific polypeptide growth factors. For example, the oncogenic virus SV40 stimulates quiescent Balb/3T3 cells to synthesize DNA, an action that was not complemented by PDGF or plasma, which indicates a viral gene function that overrides requirements for normal growth regulators. In contrast, tumor promoter phorbol esters stimulate cell proliferation by functioning as competence factors. However, TPA alone does not stimulate cell replication and thus distinguishes this compound from SV40.

Gospodarowicz and Vlodavsky emphasized the role of the extracellular matrix and growth factors in the control of proliferation of anchorage-dependent cells. Following its original proposal by Grobstein, a role for cell-substrate interactions in the control of cell proliferation and morphogenesis has been demonstrated. In vivo normal and neoplastic cells are in close contact with a basal lamina or extracellular matrix composed for the most part of collagen, glycosaminoglycans, and glycoproteins. In vitro culture of human cells on such a matrix rather than directly on a plastic surface stimulated cell growth, particularly with colon carcinoma cells, to a point that EGF was no longer mitogenic. Cells on plastic required an additional EGF mitogenic stimulus to express fully their proliferative potential. Results were presented that suggested the proliferative effect of fibroblast growth factor under certain conditions could be mediated by an influence on the formation of an extracellular matrix which, in turn, should enhance normal or tumor cell proliferation. These studies highlight the possibility that the lack of response of various cell types maintained under tissue culture conditions to agents responsible in vivo for their proliferation and differentiation is directly attributed to the artificial substrate, whether plastic or glass, upon which the cells rest and which limits their ability to produce an extracellular matrix.

The induction of differentiation of murine embryonal carcinoma cell lines by in vitro exposure to retinoic acid was reported by Strickland. Differentiation to parietal endoderm was associated with induction of collagen and laminin synthesis, plasminogen activator production, loss of embryonic antigen, and development of *H-2* expression. In vivo studies indicated that retinoic acid treatment delayed mortality after embryonal carcinoma transplantation.

Dexter, in his studies of the effect of dimethylformamide on a series of human colon carcinoma lines, found that treatment with it slowed the growth of cell lines, decreased saturation density, abolished clonogenicity in soft agar, and caused an increased expression of carcinoembryonic antigen. When human colon cancer cells were pretreated in culture with dimethylformamide and then injected into nude mice, their tumorigenicity was greatly reduced compared with their untreated counterparts. Preliminary results were presented with antimetabolite and X-irradiation therapy in conjunction with dimethylformamide, which supported the concept that a protocol in which an inducer is combined with a conventional treatment modality might prove to be a most efficacious method of cancer treatment with BRM.

Todaro discussed autocrine secretion and malignant transformation of cells in which the classic requirement of malignant cells for exogenous growth factors can be simply

explained by endogenous production of growth-promoting polypeptides by transformed cells. Of the TGF that have been isolated from a number of human and animal carcinoma and sarcoma cells, the first factor to be recognized as such was SGF, which was isolated from 3T3 cells transformed by Moloney sarcoma virus. It is a potent mitogen and causes overgrowth and morphological transformation of normal fibroblasts. Other TGF isolated from various human tumor cell lines all strongly promote anchorage-independent multiplication and colony formation of indicator cells. They strongly compete with EGF for cellular receptors that are specific for it; however, SGF is antigenically distinct from EGF and differs from it in a number of biochemical properties. As various TGF are defined chemically, synthesis of peptide antagonists is possible, as has been done with several other peptides, with a view toward use of such agents for prevention or therapy of cancer.

T. L. Low and A. Goldstein reviewed the current status of the purification and biological studies of factors with thymic hormone-like activity. Table 9-2 provides a summary of the chemical properties and biological effects of these factors. Three categories of factors may be recognized in homogenates of whole thymus tissue; one class of peptides are products of thymic epithelial cells, which should be termed thymic hormones; the second class, produced by thymocytes, are likely to be lymphokines; the third are tissue products and are not thymic specific. The most important contribution of thymic hormone research resides in its application to the clinical management of thymus-dependent diseases. Ongoing clinical trials of thymosin have suggested that it has a major role in restoring immune responsiveness and augmenting specific lymphocyte activities in children with hypothymic function and in patients with secondary T-cell deficiencies that result from various disorders, including cancer and autoimmune disease. In anergic cancer patients, thymosin or other thymic hormones may be important as adjuncts to conventional treatments by increasing T-cell function in response not only to tumor cells but also to pathogens; thus the high incidence of infection that often accompanies cancer treatment is reduced.

The development of an increasing number of in vitro assays capable to detecting monokine and lymphokine activities in the supernatant of human or cultured murine cells has led to the description of many factors. In most instances, the lack of a clear association of a purified effector molecule with a particular biological assay has made analysis extremely difficult. Recently, 2 factors that modulate the activation of human and murine T-lymphocytes have been distinguished on the basis of biochemical and biological criteria. The first of these, TCGF, also known as IL-2, was described by Ruscetti and Gallo in the human system and by Watson and Gillis in the murine system. The chemical and biological properties of IL-2 are summarized in table 9-3. Gillis reported on the identification of 2 potent producer tumor cell lines. One, designated LBRM-33, is a murine T-cell lymphoma which generates (upon stimulation with T-cell mitogens) between 1,000 and 5,000 times the amount of IL-2 present in conventionally prepared murine spleen cell-conditioned medium. A second producer line, designated Jurkat-FHCRC, is a human T-cell leukemia that generates similar high titer IL-2, which is stimulated by PHA. These lines serve as excellent sources of biochemically defined IL-2 for large-scale production and purification and will facilitate development of constitutive producing T-cell hybridomas.

Ruscetti and Gallo reported on their successful long-term growth of human leukemia cells from patients with cutaneous T-cell lymphomas (mycosis fungoides, Sézary syndrome) and E-rosette-positive T-cell ALL. Successful long-term growth of malignant T-cells was reported in 20 of 25 patients and totally depended on a source of human TCGF. Unlike long-term lines of normal T-cells, an antigenic or mitogen stimulus was not required, presumably because malignant T-cells were activated during the process of transformation. Some of the malignant cell lines originally dependent on the addition of TCGF for in vitro growth sequentially became independent of it and were constitutive producers of low levels of TCGF as well as other growth factors, such as myeloid CSA and erythroid burst-promoting activity. A unique replicating C-type virus was isolated from some of these malignant T-cell lines and may be an etiological agent in cutaneous T-cell lymphoma. Clearly, this cell system will be useful to investigators for 1) providing malignant T-cells for biochemical and virological studies relating to etiology; 2) comparative studies between normal and neoplastic T-cells; 3) possible predictive value in patients in remission by utilizing the direct response of transformed T-cells to TCGF as an indication of the presence of residual neoplastic cells. An analysis of patients with various types of leukemia and lymphoma with respect to terminal transferase activity and TCGF to determine the significance of TCGF production and response as functional markers for human leukemias was presented by Mertelsman. The positive terminal transferase leukemias revealed a markedly abnormal TCGF production and response pattern, including low mitogen-induced proliferative response, high levels of production of this growth factor, and minimal proliferation in response to added purified TCGF or that endogenously produced. The same TCGF production and response pattern was observed in 2 cell lines with positive terminal transferase activity that were derived from human lymphoblastic leukemias. These data further emphasize the suggestion that aberrant proliferation and lack of maturation observed in these leukemias are associated with or are the result of a breakdown in regulator-mediated control proliferation.

The second factor is involved in the cascade-type sequence involving several classes of macrophages, T-cells, and B-cells that result in the proliferation. Chemical characterization revealed that the immunosuppressive activity from MuSU-3T3 cells was associated with 2 disulfide-containing proteins with mol wt of 8,000 and 12,000, resolvable by gel filtration chromatography from the SGF produced by the same cells. The human immunosuppressive factors also had a low mol wt of 10,000 and were basic disulfide-containing peptides.

Hoffmann described the coordinated system of inductive and suppressive events involved in the activation of B-lymphocytes in the initiation of the humoral immune response. A synergism was reported between IL-1 and a

TABLE 9-2.—*Thymic preparations and their biological effects*

Thymic preparations	Principal investigators	Chemical properties	Biological effects
Thymus tissue			
Thymosin fraction 5	Hooper JA, Goldstein A, et al.	Family of heat stable, acidic polypeptides; mol wt, 1,000–15,000	Induces T-cell differentiation and enhances immunological function in animal models and in humans
Thymosin α_1	Low TLK, Goldstein A, et al.	Polypeptide of 28 residues; mol wt, 3,108; pI 4.2;[a] sequence determined	Increases mitogenic responsiveness of murine lymphocytes; induces enhancement of MIF production; increases Thy 1.2 and Lyt 1,2,3 positive cells and helper T-cells; modulates TdT activity
Thymosin β_4	"	Polypeptide of 43 residues; mol wt, 4,982; pI 5.1; sequence determined	Induces terminal transferase in vivo and in vitro in bone marrow cells from normal and athymic mice; induces this enzyme in vivo in thymocytes of immunosuppressed mice
Thymosin α_7	"	Acidic polypeptide: mol wt, 2,500; pI ~3.5	Enhances suppressor T-cells in vitro; expresses Lyt 1,2,3 positive cells
THF	Trainin N, et al.	Polypeptide; mol wt, 3,200; pI 5.7; amino acid composition determined	Restores ability of spleen cells from neonatally thymectomized donors to induce an in vivo GVH reaction; enhances ability of normal spleen cells to respond to PHA and Con A
Thymopoietin	Goldstein G, et al.	Polypeptide of 49 residues; mol wt, 5,562; pI 5.5; sequence determined	Causes delayed impairment of neuromuscular transmission in vivo; induces bone marrow cells to develop intrathymic lymphocytes
Thymic polypeptide preparation	Milcu SM, Potop I, et al.	Mixture of polypeptides and amino acids	Elevates serum calcium concentration and decreases serum inorganic phosphate; stimulates antibody synthesis in immunized, X-ray-irradiated rabbits
Thymosterin	Potop I, et al.	Lipids or lipoproteins, one purified component (IIB_3) a C_{28} steroid with methyl groups at C-21 and C-28	Has antiproliferative action on KB tumor cells in culture; repairs the specifically altered metabolism of normal or thymectomized tumor-bearing animals
Blood			
FTS	Bach JF, Dardenne M, et al.	Nonapeptide; mol wt, 957; pI, 7.5; sequence determined	Enhances generation of effect of cytotoxic T-cells in vitro and in vivo; inhibits contact sensitivity in normal mice
Protein fraction from human plasma	White A, et al.	Protein; mol wt, 57,700; properties analogous to prealbumin	Enhances ability of neonatally thymectomized mice to reject an allogeneic skin graft; enhances MLR of mouse spleen cell ineubated with the blood fraction
Human SF	Astaldi A, et al.	Adenosine (possible other small molecular weight components)	Increases the intracellular cAMP levels of human and mouse thymocytes; increases the population of hydrocortisone-resistant cells
Thymic epithelial supernatants			
Thymic epithelial supernatant	Kruisbeck AM, et al.	Crude extract, chemical nature not characterized	Augments the proliferative responses of rat thymocytes to PHA or Con A; stimulates mixed lymphocyte reactivity and antibody production to SRBC by spleen cells from athymic mice
Human thymic epithelial medium	Kater L, et al.	Crude extract, chemical nature not clear	Augments the mitogen responsiveness of thymocytes
TFX	Aleksandrowicz J, Skotnicki AB, et al.	Polypeptide; mol wt, 4,200; amino acid composition reported	Restores azothioprine sensitivity of spleen rosette-forming cells from adult thymectomized mice in vitro; increases blood T-cell number and return of DTH hypersensitivity in vivo
Thymostimulin	Falchetti R, et al.	Mixture of polypeptides	Induces markers and specific functions of T-lymphocytes in immunosuppressed animals and in immunodeficient patients; stimulates IF production in mice after challenge with poly I:poly C

TABLE 9-2.—*Thymic preparations and their biological effects (continued)*

Thymic preparations	Principal investigators	Chemical properties	Biological effects
Porcine thymic preparation	Jin Y, et al.	Mixture of polypeptides; mol wt, 9,000–68,000; pI, 5.0–7.5	Increases rosette formation in fetal thymocytes
Bovine thymic preparation	Liu SL, et al.	Mixture of polypeptides	Increases rosette formation in umbilical cord blood lymphocytes
Homeostatic thymic hormone	Comsa J, et al.	Glycopeptide; mol wt, 1,800–2,500	Suppresses deleterious consequences of thymectomy in young guinea pigs; restores DTH of thymectomized rats
Lymphocytopoietic factors (LSH_h and LSH_r)	Luckey TR, et al.	Polypeptides; mol wt, 80,000 (LSH_r), 15,000 (LSH_h); amino acid composition of LSH_r available	Enhance antibody production to SRBC and induces lymphocytosis
Hypocalcemic and lymphocyto-poietic substances (TP_1 and TP_2)	Mizutani A, et al.	Polypeptides	Enhance production of antibody to SRBC in neonatal mice; hypocalcemic activity in normal rabbits

[a] pI = isoelectric point.

TABLE 9-3.—*Properties of human and murine IL-2 (TCGF)*

Chemical and biological properties	IL-2		
	Human	Murine	Rat
Size (gel filtration)	13,000	30,000	15,000
Elution from DEAE ion exchange resin, pH 7.6	0.05 *M* NaCl	0.15 *M* NaCl	0.05 *M* NaCl
Isoelectric points	6.8	4.3–4.9	5.4–5.5
Stable in pH range	2.0–9.0	2.0–9.0	2.0–9.0
Absorption of activated murine T-cells	Yes	Yes	Yes
Stimulation of growth of murine T-cell lines	Yes	Yes	Yes
Proliferation of murine thymocytes stimulated in presence of Con A or PHA under culture conditions and with limiting mitogens (alone)	Yes	Yes	Yes
Generation of cytolytic lymphocytes in murine thymocyte and nude spleen cultures	Yes	Yes	Yes
Stimulation of antibody responses to heterologous erythrocyte antigen in nude spleen cultures	Yes	Yes	Yes
Cell of origin a subset of activated T-lymphocyte (distinct from target T-cell)	Yes	Yes	Yes

35,000-mol wt mediator released by helper T-cells (TRF) in the induction of antibody-forming B-cells. Experimental studies in cultures of mouse spleen cells induced to produce antibody plus RBC antigen suggested that T-cells provided a signal, TRF, necessary for the terminal differentiation of B-cells into antibody-secreting cells. Only a small fraction of B-cells is mature enough at any given time to respond to that signal. That fraction is increased in response to another signal, IL-1, which induces earlier stages of B-cell differentiation. A model of B-cell activation was described in which antibody formation may be enhanced or suppressed by helper factors (IL-1 and TRF) depending on the sequence of helper factor addition. If a TRF is added after 2 days, the predominant effect is inductive as the TRF acts to stimulate a pool of IL-1-recruited B-cells to produce antibody. If the TRF is added before day 2, before most of the B-cells react to it, the response is inhibited as the combined action of the TRF and IL-1 induces suppressor cells. In this way, macrophages and T-cells can synergize to stimulate the antibody response, while at the same time they can prevent excessive antibody formation by the generation of suppressor B-cells.

A description of the differentiation heterogeneity in murine hematopoietic tumors, particularly those of the B-cell lineage, was provided by Warner. One can recognize intertumor heterogeneity. Generally, researchers have con-

cluded that there are at least 4 major types of B-cell tumors: 1) pre-B typified by intracellular synthesis of Ig but lack of secretion or membrane expression of Ig; 2) virgin B-cells which express membrane Ig but fail to activate J chain synthesis and hence secretion of polymeric IgM; 3) activated B-cells which secrete Ig; and 4) plasma cells of varying stages of maturity. Intratumor heterogeneity also can be demonstrated by the use of biophysical cell separation, cell surface markers, and functional properties showing that not all cells need be alike within a cloned tumor cell population. In this context, selection for the tumor stem cell population as distinct from the more mature cells is possible. One of the most interesting aspects of tumor heterogeneity is whether this can be deliberately manipulated by experimental procedures. As such, this could have direct clinical relevance if true terminal maturation could be induced. This was illustrated by the effect of the phorbol ester TPA on the B lymphoma 2PK-3 that induced the population as a whole toward the direction of more mature Ig-secreting cells with reduction in surface Ig, Ia, and Fc receptors and considerable depletion of clonogenic cells. More differentiated function in the myelomonocytic tumor WEHI-3 was also induced by TPA.

Ralph also reported on differentiated functions in lymphoid and macrophage tumor cell lines. Lectins such as Con A and PHA are toxic to most mouse and human hemic cell lines but can induce the expression of new products by the cancer cells. For example, T-cell lymphomas can be induced to secrete IL-2, myeloid CSF, and megakaryocyte-stimulating factors. Similarly, the B-cell mitogens dextran sulfate and LPS are cytostatic to B-cell lymphomas but induce surface Ig and Fc receptors. The phorbol ester is also a strong inducer of Ig secretion by human B-cell lines. Murine macrophage tumor lines could also be induced by agents, such as LPS, BCG, and phenylmyristic acetate, to secrete myeloid CSF, prostaglandin, and plasminogen activator, and additionally acquire enhanced functional properties, such as phagocytosis, antibody-dependent killing of RBC and tumor targets, and direct cytotoxicity to tumor targets. In a study of the stimulating effects of macrophage activators on cell lines, the simultaneous presence of hydrocortisone or dexamethasone could reverse these actions.

Gallo described the features of the human HL-60 promyelocytic leukemia cell line developed in his laboratory. A small percentage of the HL-60 cells spontaneously differentiate to more mature myeloid cells, and DMSO markedly enhances terminal differentiation of HL-60 to polymorphs with the capacity to generate superoxide; reduce nitro blue tetrazolium; phagocytize and kill microorganisms; and respond to chemotactic factors with the presence of complement receptors, increased hexose monophosphate shunt activity, and increased release of packaged enzymes. A wide variety of compounds other than DMSO induce HL-60-differentiation: These include dimethyl formamide, hexamethylene bisacetamide, butyric acid, hypoxanthine, dactinomycin, and retinoic acid. The findings with retinoic acid prompted an investigation of the sensitivity of this inducer of human myelocytic leukemia cells in short-term primary suspension culture. Of 21 patients, only cells from the 2 with acute promyelocytic leukemia differentiated in response to retinoic acid, which suggested the probable clinical value of retinoid therapy in this leukemia variant. Phenotypic macrophage-like alterations of HL-60 were seen when the cells were incubated with a source of CSA in suspension culture. Both Gallo and Rovera reported that low concentrations of the TPA added to HL-60 caused various rapid changes. The cells lose their granules, become adherent, spread, cease proliferating, and develop the ability to phagocytize latex, IgG-coated RBC, and *Candida.* The percentage of cells bearing Ig or C3b receptors increases, and cells become positive for alpha-napthyl acetate esterase, a marker for monocytes and macrophages. In a study of 60 patients with leukemia, Rovera described a consistent pattern of in vitro response to TPA in acute myeloblastic and myelomonocytic leukemia with appearance of macrophage markers. Undifferentiated leukemias were heterogeneous; some responded to TPA treatment, whereas others did not develop any macrophage differentiation markers. In the range of 10^{-8}–10^{-9} *M*, TPA will prevent the formation of colonies of leukemia cells in vitro, which suggests that treatment with it is indeed efficient in arresting cellular proliferation of leukemia cells. In vivo treatment of mice with TPA, including nude mice bearing the hematopoietic tumor K562, suggested that the concentration of TPA capable of optimum differentiation–induction is close to the toxic dose for the animal. However, in vitro treatment of leukemic marrow with TPA followed by autologous transplantation is a viable possibility, inasmuch as TPA does not appear to influence the pluripotent stem cell.

Golde reported on 2 other human leukemia cell lines, KG-1 of myeloid leukemic origin, and Mo, a T-cell line obtained from a patient with hairy cell leukemia. This latter line produces various hematopoietic growth factors, including myeloid CSF, erythroid potentiating, mixed CSF and MAF.

A new assay for measurement of self-renewal of human leukemia blast progenitors as a target for therapeutic intervention was deseribed by Buick. Conventional clonogenic assays for human myeloid leukemia cells have allowed the testing of agents, such as cytotoxic drugs, on primary plating efficiency. By recloning primary colonies one can now measure a different index, i.e., the replating efficiency (PE-2) which is a possible measure of leukemia cell self-renewal. The colony assay is based on stimulation of peripheral blood leukemia blast populations depleted of T-cells with a PHA–leukocyte-conditioned medium in a methylcellulose matrix. A highly significant correlation was found between concentrations of morphologically identifiable blast cells in the peripheral blood and colony formation at diagnosis. The results are consistent with the view that a minority of leukemia blasts have sufficient proliferative capacity to be clonogenic and represent the population maintaining the tumor in situ. A significant correlation was seen between a low self-renewal index (PE-2) and successful remission induction. By far the most dramatic changes in self-renewal capacity of AML progenitors has been brought about by agents thought to act at the level of control of differentiation. After treatment with tumor promoters, the low self-renewal index was increased

in surviving colonies, whereas low doses of human fibroblast IF inhibit it, and ara-C also decreases it.

Price, Senn, and Gorczynski reviewed their studies that indicate a significant similarity and association of various molecular weight species of macrophage products which stimulate myeloid and lymphoid cell populations. In the mouse, these macrophage products, at least for the lymphostimulatory function, were demonstrably MHC restricted. Affinity chromatography with anti-β_2-microglobulin removes up to 90% of the activity in macrophage culture supernatants capable of inducing a T-lymphocyte cytotoxic response and up to 100% of the myeloid CSA. Analysis of the molecular weight heterogeneity of CSF produced by leukocytes of patients with various diseases showed that patients with untreated acute or chronic leukemia or acute leukemia in relapse generally produce a single species of 36,500 daltons. Patients with nonmalignant disorders typically show 3 molecular weight species. A high correlation was seen between restricted release of a single CSF species by leukocytes from patients with preleukemia syndromes and subsequent progression to acute leukemia.

Shadduck, using radioiodinated CSF purified from mouse L-cell conditioned medium reported on his binding and autoradiographic studies. Using density gradient separation of normal mouse marrow or fetal liver cells, he detected that CSF binds to both proliferating and nonproliferating cells. Autoradiographs show that, after 6 hours of incubation, binding to bone marrow and fetal liver cells is confined to large mononuclear cells and proliferative elements of the granulocyte series. Although the CSF is believed to function primarily as a regulator of granulocyte and macrophage production, several observations suggest that this material may also act on more mature cells of the GM series. This factor induces RNA synthesis in suspension of mature neutrophilic granulocytes and specifically activates mature macrophages to induce tumoristatic and tumoricidal activity. In contrast to the action of insulin and related hormones in which cellular binding occurs at 4°C with maximum binding over 15–60 minutes, virtually no CSF binding occurs at reduced temperatures. Thus CSF binding appears to require both the synthesis of binding protein as well as the specific interaction of CSF with GM cells at all stages of maturation.

Lord and Wright summarized these studies on endogenous stem cell proliferation regulators. The potential therapeutic value of this area relates to the fact that damage to hematopoietic stem cells is one of the most important considerations when cytotoxic therapy procedures are developed, particularly when the therapy is administered in a fractionated manner over a designated period. Endogenous factors are readily isolated from hematopoietic cell suspensions and are partially purified by ultrafiltration. The fraction with a nominal mol wt of 50,000–100,000 protected rapidly proliferating murine stem cells (CFU) from the lethal effect of a large dose (200 μCi/ml) of [^{3}H]dThd. Conversely, an extract of 30,000–50,000 daltons obtained from regenerating bone marrow stimulated slowly proliferating CFU into DNA synthesis, leaving them sensitive to the killing effect of high doses of [^{3}H]dThd. The proliferative status of CFU is determined by the balanced endogenous production of these two opposing activities within the hematopoietic tissue. The proliferation regulators are specific for multipotential stem cells and do not influence the cycle status of committed myeloid and erythroid progenitors. The stimulatory and inhibitory regulators do not appear to be species specific, and human bone marrow, fresh or grown in long-term culture for 2–6 weeks, also produces the regulators in equivalent proportions to other sources.

A cell contact modulation of granulopoietic proliferation and differentiation mediated by adherent mononuclear cells was described by Spitzer and associates, who noted inhibition of myeloid leukemia cell cloning after a short preincubation of leukemia marrow cells with normal adherent mononuclear cells. Specificity for inhibition of leukemia target cells was indicated because normal allogeneic bone marrow cloning was resistant to inhibition following an identical preincubation with adherent cells. Inhibition of leukemia cells was due to a direct cell-to-cell contact mechanism rather than to production of a diffusible growth inhibitory soluble factor. Furthermore, inhibition was directed at the clonogenic subpopulation of leukemia cells and, as such, the effect may not be detected in a cytotoxicity assay such as ^{51}Cr release.

Verma reported on the effects of human leukocyte IF preparations on granulopoiesis in vitro. (Therapeutic administration of IF has been associated with granulocytopenia.) Leukocyte IF produced a dose-related decline in human myeloid colony size with a corresponding increase in cluster size aggregates. These opposing effects resulted in an almost constant plating efficiency regardless of the IF dose. Morphological examination of the cultures revealed a dose-related granulopoietic differentiation block. The growth stimulating factor competitively counteracted the IF effect. Human fibroblast IF seemed to be much less active in blocking granulopoietic maturation, whereas human immune IF was as active as that in leukocytes. These data suggest a regulatory role for leukocyte IF in granulopoiesis.

Moore discussed the linked production of a wide spectrum of hematopoietic growth regulatory factors by the WEHI-3 murine myelomonocyte leukemic cell line, a spectrum duplicated by lectin-stimulated normal spleen cells. The feature of the WEHI-3 cell is its ability to produce factors constitutively and thus circumvent control networks implied by mitogen–lymphocyte–macrophage interactions. The cell line produces distinct M-CSF, G (neutrophil)-CSF, GM-CSF, eosinophil-CSF; megakaryocyte-CSF, erythroid burst-promoting activity, IL-1, endogenous pyrogen, mast cell growth factor, neutrophil stem cell, pluripotent stem cell stimuli, and PGE. It retains the ability to differentiate to macrophages and granulocytes when exposed to purified GM-CSF or to a differentiation protein of 28,000 daltons isolated from postendotoxin serum. Normal myelomonocyte progenitor cells respond to GM-CSF but do not produce it; in contrast, the neoplastic myelomonocytic cells both produce and respond to GM-CSF. The presence of CSF receptors on the cell surface associated with growth factor production represents an opportunity for autostimulation. A genetic defect in response to 2 of the growth factors produced by the cell

line, G-CSF and mast cell growth factor, was reported in NZB, NZC, and C58 mice. This may be due to a defect in the CSF molecule, i.e., WEHI-3, G-CSF, or mast cell growth factor. This latter factor is a leukemic product similar but not identical to normal G-CSF. Alternatively, the impaired response may indicate multiple defects in G-CSF-unresponsive mouse strains variously involving autoimmunity, endogenous xenotropic virus expression, and high leukemia incidence.

WORKSHOP ON THE ROLE OF MODIFIERS ON MACROPHAGES, NATURAL KILLER CELLS, AND ANTIBODY-DEPENDENT CELLULAR CYTOTOXICITY IN TUMOR REJECTION

A considerable amount of new and pertinent information was presented at this workshop concerning the characteristics of cytotoxicity against a variety of human and animal tumor cells by macrophages, NK cells, and ADCC.

Stewart reported on the activation and killing kinetics of macrophages exposed to lymphokine-rich medium. Activation of macrophages to a tumoricidal state required 12–16 hours; once the tumoricidal state was reached, macrophages lost this activity within 16 hours. Of particular importance was that macrophages do not have to interact continuously with the target to cause lethal damage. Macrophage–tumor cell interaction during the first 12 hours after activation was sufficient. Oppenheim described the means by which such macrophage–lymphocyte interactions lead to the generation of cytokines, such as IL-1, CSF, and IF. These endogenous signals activating lymphocytes and promoting the expression of macrophage Ia and FcR enhance the capacity of these cells to maintain host integrity. Information was given that supported the concept that a multistep mechanism is required for activating macrophages to express cytolysis. Both Meltzer and Russell presented evidence that LPS is essential as a second signal. For the activation of mouse macrophages by an activating factor, LPS acted synergistically with lymphokines to optimize macrophage tumoricidal activity. From mouse studies, it does not appear that a second signal by LPS is required for activation of macrophages by IF or IF inducers. This is an important point because some of the BRM under consideration for testing may be expected to activate macrophage tumoricidal activity through the IF pathway. Mantovani and LoBuglio stated that they found no evidence for LPS serving as a second signal for activation of human monocytes. LoBuglio reported results showing that, if monocyte cell suspensions were incubated with various doses of human fibroblast IF for 18 hours and then placed in coculture with tumor cells, a consistent enhancement of cytotoxicity was achieved.

Several presentations were concerned with the induction of, and possible mechanism for, suppressor activity macrophages and the important role of PGE. Bennett reported on the tissue distribution, phenotypic characteristics, and pharmacological regulation of suppressor cells induced by systemic administration of BCG. Indomethacin, salicylate, aspirin, and Ro 3-1314 added to the initiation of coculture with BCG-induced suppressor cells, and normal lymphocytes were reported to block the inhibition of alloimmunization produced by these suppressor cells. The BCG-induced splenic suppressor cells were considered to be immature macrophages. Bennett also provided evidence concerning the ability of prostaglandins to inhibit the generation of cytotoxic cells. Russell and Schultz presented evidence that exogenously applied or endogenously produced PGE_2 modulated the cytotoxic activity of macrophages or the maintenance of the activated state, or both. Of particular interest concerning the regulating function of PGE_2 on macrophage cytotoxicity was the observation that LPS, though capable of activating macrophage tumoricidal action, also causes production of prostaglandins in quantities sufficient to limit the optimum macrophage activation. In contrast, IF, capable of directly activating macrophage cytotoxicity, does not markedly stimulate PGE production and thus limits the negative feedback inhibition of macrophage cytotoxicity exerted by PGE. Bonney reported that macrophages from mice treated with BCG or *C. parvum* release less of the prostaglandins than do resident macrophages. These changes were accompanied by those characteristic of elicited cells, such as in the activities of 5′-nucleotidase and leucine aminopeptidase. In contrast, Drysdale and Shiu presented evidence for augmentation of macrophage activity for PGE in the ADCC response; also, the prostaglandin further demonstrated its role in the enhanced macrophage cytolytic activity elicited by human chorionic gonadotropin. Papademetriou reported that macrophages from mice treated with this hormone suppressed normal splenic lymphocyte response to PHA. This suppression was considered to be due to the high concentration of PGE_2 elaborated by macrophages exposed to the hormone. Hibbs determined that perturbation of energy homeostasis, in the form of deranged oxygen metabolism in L1210 leukemia cells and transformed cells, was one mechanism by which activated macrophages exert their cytotoxic effect. Results of 3 studies indicated that the application of immune modifiers elicited a therapeutic response by the way of macrophages. Muchmore, using a unique spontaneous monocyte-mediated cytotoxicity assay, presented data that suggested X-irradiation and *cis*-dichlorodiammineplatinum(II), acting by different mechanisms, result in marked in vitro activation of human monocyte-mediated cytotoxicity. Irradiation appeared to have a primary effect on a lymphocyte suppressor cell population, whereas the antineoplastic agent appeared to act directly on activating monocyte cytotoxicity. Chirigos reported on the multifaceted characteristics of several BRM which were shown capable of eliciting more than one of the effector cell mechanisms. Several were capable of enhancing both macrophage and NK cell activity; many of these agents are IF inducers, but a few were active only on B-cells. Data from in vitro and in vivo studies demonstrated the effector role that macrophages play in retarding tumor growth. A strong synergistic antitumor effect was shown when BRM, such as MVE-2 and poly ICLC, were combined with tumor cytoreductive chemotherapy. Mantovani determined that tumor-associated macrophages could be enhanced by endotoxin, IF, or lymphokines in their cytolytic activity. His evidence indicated that not all tumor-associated

macrophages from autologous ovarian carcinomas can be augmented by IF or lymphokines and that IF enhanced the cytotoxicity of PBL and tumor-associated lymphocytes.

The importance of NK cells was discussed on the second day of the workshop first by Herberman, who presented a comprehensive review of the state of the art about NK cells. He described the characteristics of NK cells, indicating them to be a subpopulation of lymphocytes expressing T-cell associated markers and receptors for the Fc portion of IgG. Through the use of various separation techniques, Herberman found that the NK cell activity is associated with large lymphocytes with an indented nucleus and prominent azurophilic granules in the cytoplasm (large granular lymphocytes). The results of Herberman and Djeu indicated that NK cells express augmented cytotoxic activity after exposure to IF; activity was demonstrated within 1 hour after exposure, and IF was bound to NK cells within 5–10 minutes at either 4° or 37° C. The mechanism by which IF augments NK cells appears to be by acceleration of the rate of lysis by already active NK cells. The large granular lymphocyte was identified as possessing NK cell activity. In addition, Djeu reported that the former cell possesses spontaneous NK activity which was boosted in the presence of IF inducers. The suggestion was made that NK cells have multiple natural host defense functions and can produce IF in response to various biological agents as well as lyse a wide range of target cells. It was considered highly likely that NK cells have an important function not only in resistance against tumors, but, as discussed by Cudkowicz, in natural resistance against bone marrow transplants and possibly in the regulation of normal cell differentiation.

Santoli described an MLC assay that was considered capable of generating cytotoxic cells from inactive precursors. The data presented indicated that the toxic cells generated in the MLC are newly generated from precursors contained in the responder FcR-negative, E-rosette-forming cell population. She emphasized the potential utility of the MLC assay as an in vitro model for the study of the regulation of cell-mediated defense mechanisms and the role of IF. Results on target cell lysis by cloned cell lines with NK cell activity were discussed by Dennert who had initiated permanent cell lines from mouse spleen cells. He cloned one of the cell lines by limiting dilution and assayed it on several mouse and human NK-sensitive targets to determine whether target specificities segregate upon cloning. Target specificities of the cloned lines were identical to those of NK cells in normal spleen. He considered it likely that NK cells recognize identical target structures on all NK-sensitive targets. His conclusions were that lysis of NK targets is a function of two variables: effector binding to the target and sensitivity of the target to lysis. Cell surface marker analysis of NK cells reveals that they are Thy 1.2^+, Lyt 1^-2^-, $T200^+$, asialo $GM1^+$, asialo $GM2^+$, which distinguishes them from specific cytolytic thymus-derived lymphocytes.

Zarling emphasized the augmenting activity of human fibroblast IF on human cytotoxic T-cell and NK cell activities. Lymphocytes from several normal individuals were shown to lyse fresh human leukemia cells only after treatment with IF. Several structural analogs of polyrIn·rCn were examined for their augmenting activity on NK cells; only those that induced IF were effective. She demonstrated that monoclonal antibodies could distinguish cytotoxic T-lymphocytes from NK and NK-like cells.

According to Einhorn, IF-β and IF-α enhanced NK cell activity against Chang and K562 cell lines. Exposure of NK cells to multiple pulses of IF demonstrated an increase in response. In contrast, target cells exposed to IF were less susceptible to NK cells. Peripheral blood from patients treated with IF showed augmented NK cell activity at 24 hours after treatment. The NK cell activity remained elevated in patients who received IF therapy for 3 months. In a review of suppressor mechanisms and cellular interrelationships in cancer, Allison presented possible mechanisms of cytotoxicity by macrophages that would include the effects of oxygen radicals, proteases, or C_3a. He suggested that the primary effect of IF may be to alter states of differentiation of various cells. Of particular interest was the potential role of polyamine oxidase in regulating macrophage activity.

Among the various mechanisms of the ADCC response described on the third day of the workshop was the effect of ara-C on ADCC response and Fc receptor cells discussed by Zighelboim. With the ADCC assay, ara-C selectively modulated the function or structural constituents, or both, of lymphoid cells. The data presented suggested that ara-C could effectively and selectively influence K-cells. As summarized by Koren, little evidence is shown for a role for endogenous ADCC in vivo. This was considered related largely to the difficulties in our distinguishing between the direct cytotoxic effects of various effector cells and the possible involvement of such cells in ADCC. Pollack stated that the suppressors of ADCC in the murine system are T-cells and that their effect was not competitive but mediated by the cell surface. Huard used a rabbit model to examine the interaction of peripheral blood monocytes and tissue-bound alveolar macrophages with tumor cell targets sensitized with rabbit antibody. Alveolar macrophages were reported to be highly effective at binding antibody-coated tumor cells, whereas peripheral monocytes were much less effective. These results suggest that criteria for Fc receptor interaction with antibody-sensitized target cells can differ dramatically at different stages of differentiation in the monocyte–macrophage cell lineage. In vivo treatment with complete Freund's adjuvant, BCG, and MDP resulted in a significant increase in ADCC response in peripheral monocytes and alveolar macrophages.

WORKSHOP ON HYBRIDOMAS IN THE DIAGNOSIS AND TREATMENT OF CANCER

Over the course of 2.5 days, 16 distinguished investigators presented their research with this exciting new advance in immunological technology. During the 30-minute discussion periods, a number of investigators presented their complementary data on the subject of each talk, with enlightening exchanges of information and concepts.

Lennox began the meeting by putting hybridoma technology into perspective. The merits of xenogeneic hybridoma products, specifically mouse anti-human mono-

clonal antibodies, versus human anti-human monoclonal antibodies were discussed. Although the latter antibodies are far more likely to identify what human lymphoid cells "see" on the surface of human tumor cells or on human altered self-components, they are far more restricted than mouse antibodies, which can effectively identify a broader range of antigenic specificities on the surface of human cells. Thus one or the other type of monoclonal antibody may be preferable in a set of circumstances, but neither should be considered generally more desirable without further examination. Identification of antigens on tumor cells and determination of whether any are unique to the tumor should be facilitated by monoclonal, highly specific antibodies, many of which are directed to limited parts of the cell membrane. On the other hand, one must now strive to avoid the mistake of calling 2 epitopes on the same antigen different antigens, which is a pitfall with antibodies of such a fine discriminatory capacity. References were made by speakers throughout the conference to Lennox's statement that the question: "How specific is the antibody?" should be met by: "Why do you want to know?" In other words, the purpose for which one wants to use the antibody determines the degree of specificity one requires in that reagent. Diagnosis of a tumor through detection of antigens in the serum may require only that the antibody react with that antigen even if it reacts with other substances, as long as the others are not found in the serum. On the other hand, if one uses the reagent to localize a tumor in normal tissue, specificity is critical, as it is when toxic agents are to be targeted by specific antibody and thereby brought to the site of the tumor.

Gefter illustrated the information that can be obtained through the use of monoclonal antibodies as probes into the genetics of antibody production. Even though the same V_H gene is ultimately responsible, the idiotype is unique to each of a variety of monoclonal antibodies in the strain A mouse when elicited against a simple hapten, azophenylarsonate. Moreover, by immunizing rabbits with one monoclonal antibody and absorbing all of the others (15 in Gefter's experiments), one can detect a specific amino acid sequence in the idiotype that indicates a reaction to a specific antigenic determinant. One can thus monitor the inheritance of V_H genes by detecting individual sequences. It is also apparent that somatic mutations are occurring frequently to account for the variability in products one observes from the same V_H gene in the A mouse. Production of a monoclonal antibody can now be induced in tissue culture by administration of nanogram amounts of monoclonal antibody made against it; deletion of a specific monoclonal antibody is also possible by administration of larger amounts of antibody. If the theory of complementarity of each successive product to the preceding one is correct, then one can suppose that if you make an anti-anti-idiotype antibody, one might induce the idiotype without using the original antigen! In fact, this is the case. Gefter performed such experiments and obtained the idiotype after immunization with anti-iodiotype in Freund's adjuvant. Cloning of the V_H gene is now possible, and recombinant DNA technology can be used in the future to make the antibodies one wants, thereby expanding hybridoma technology still further.

Hellström presented the only paper on T–T hybridomas at the meeting, but one that will be discussed at length in a later workshop. A T-cell factor of 56,000 daltons, binding specifically to antigens of a particular MCA-induced tumor, has been identified as a blocking factor specific for that tumor. The T–T hybridomas producing the product have been made from BW 5147 cells and thymocytes on tumor-bearing mice that suppress T-cells responding to MCA 1490 but do not affect syngeneic or allogeneic lymphoid cells. The factor also enhances the growth of MCA 1490. The response of T-cell mitogens or the ability of T-cells to be immunized to LSTRA (Moloney leukemia virus tumor) all were unaffected by the suppressor factor. Footpad swelling (DTH) in response to MCA 1490 was inhibited by the suppressor factor, but the response to other MCA tumor cells was unaffected. The relationship of this hybridoma-produced factor to others, such as those elicited by Con A, is thus far uncertain, as is the exact target of the produet.

Schlossman reviewed his work with antibodies to cells of the human T-cell series, comprising early thymocytes, mature thymocytes, and peripheral T-cells. Nomenclature varies among investigators, but with Ortho-Kung T-reagents and his monoclonal antibodies, Schlossman described T-9 and T-10 markers on early thymocytes, and T-10, 6, 3, and 5 (also known as 8) markers on cortical thymocytes; T-10, 1, 3, 4 are on a subset of medullary thymocytes, with T-10, 1, 3, 5 on a second subset. The latter subsets mature into T-1, 3, 4 inducer (helper) cells and T-1, 3, 5 cytotoxic suppressor cells in the peripheral compartment. The acute and chronic T-cell leukemias have been categorized into derivatives of one or another of the stages of T-cell maturation by identification of similar markers on their surfaces. For example, the majority of T-cell ALL are T-9, 10 positive, whereas most T-cell CLL and Sézary cells are T-1, 3, 4 positive. Peripheral B-cells express B-1 antigen and more weakly B-2 antigen, but the bone marrow is mostly B-1. These are not conventional components of the B-cell surface, such as Ig or receptors for Fc or complement. Similar in weight to Ia(DR) antigen, B-1 is not on macrophages; B-1 and B-2 are expressed on most CLL cells, but only B-1 is present on common ALL antigen-positive non-T-cell ALL, and only in 50% of those. Both B-1 and B-2 are lost as the B-cell matures into a plasmacyte. These monoclonal antibodies should continue to prove useful in classification of subtypes of malignant lymphoid cells, but minor changes in glycosylation of surface components, to which monoclonal antibodies are perhaps even *too* sensitive, might obscure similarities among the various types of cell while emphasizing differences. In addition, the caveat that antibodies might be defining different epitopes rather than different markers must be kept in mind here as in any other such study.

With mouse anti-T-cell monoclonal antibodies and the fluorescence activated cell sorter, Ledbetter found that Lyt antigens increase in density as T-cells mature in the thymus and move into the medulla from more peripheral zones. Lyt 1^+ cells comprise nearly all thymocytes, 34% of all spleen cells, and 59% of lymph node cells, whereas Lyt 2^+ cells make up 82% of thymocytes, 11–13% of spleen cells,

and 20% of lymph node cells. Thus most but not all thymocytes are Lyt 123 cells, with 15–20% Lyt $1^{+}23^{-}$. Although Thy 1.2 is on all thymocytes, it decreases to 30% of spleen cells and is represented on 57% of lymph node cells. On these peripheral cells, the density of Thy 1.2 decreases by one-third that of thymocytes. In AKR leukemia, serial transplantation to mice congenic for the Thy 1 antigen causes the Lyt 1 to increase in density, but Lyt 2 becomes less dense and ultimately disappears. Ledbetter also discussed results other investigators had with monoclonal antibodies. Anti-human T-cell antibody called "Leu-1" (=SK 7) is present on all peripheral blood T-cells and is the homolog of murine Lyt 1. Although Leu 2a is on the cytotoxic-suppressor cell lineage, its density is increased on the peripheral blood T-cells relative to the density in the thymus; it is distinctly different from Lyt 2 in this respect.

During the discussion of this paper several important points were made. A participant cautioned that weak fluorescence staining of cells by an antibody to a possible surface marker cannot rule out the presence of that antigen on those cells. By the same token, strong staining shows the presence of the antigen but does not necessarily correlate with increased density. The amount of the marker not the density most influences its identification by the sorter. Fluorescence intensity varies during the cell cycle.

Hansen presented information on his mouse anti-human T-cell monoclonal antibodies. Clone 9.3 antibodies identify 60–80% of human peripheral blood T-cells, 25–56% of thymocytes, but virtually none of the non-T-cells or bone marrow cells; the 9.3 positive cells are similar but slightly more extensive in distribution than are Schlossman's T4 positive cells, and most of them are likewise helper T-cells. Obversely, the 9.3 negative population has strong suppressor activity. Blast-forming units for erythrocytes, the earliest precursor of RBC, have 0.3 reactive markers on their surface and thus appear to be related at this early level of ontogeny to the T-lymphocyte lineage. Other monoclonal antibodies useful in the identification of T-cells include clones 9.6 and 10.2. In particular, the former clone reacts with more than 99% of E-rosette-forming cells and also with more than 90% of NK cells. Ortho-Kung monoclonal antibody which reacts primarily with monocytes and macrophages, decreases NK cells akin to both the lymphoid and myeloid series. Although 10.2 and 9.6 react with T-cells, competitive binding assays show that they attach to different sites.

In his description of his experiments and those of others with antibodies against the MHC in the mouse, Frelinger stated that anti-Ia antibodies were found in the skin on Langerhans cells, which are a type of macrophage. However, Ia is also found in the white matter of the brain, probably on oligodendroglial cells, and Ia antigens are expressed on melanoma cells. In the discussion, Ferrone pointed out that neuronal cells lacked Ia and, whereas only one-third of the 24 carcinomas tested had Ia-like molecules, 20 of 28 melanomas expressed them. This Ia was similar to B-cell Ia, but it was lacking entirely in normal melanocytes. A debate ensued over whether the Ia(DR) antigens were "alien histocompatibility antigens." Several discussants mentioned that the only Ia(DR) antigens present were coded for by the genome of the individual, and thus they were not truly alien. For instance, Pollack found 45 of 50 melanomas tested had the number of these antigens expected on the basis of allotyping.

Springer reported work with anti-macrophage monoclonal antibodies, i.e., M1/79 (Mac-1), which identifies 2 protein bands of 95,000 and 195,000 daltons, respectively, that are not found on spleen cells in mice. Granulocytes in peritoneal exudates are also stained, which indicates a myeloid antigen rather than a macrophage-specific differentiation antigen. Blood monocytes stain dimly for Mac-1 antigens, but the Mac-1 positive cells are enriched for NK activity and ADCC. This monoclonal antibody against a mouse determinant also reacts against human cells. Various antibodies in the anti-macrophage series have been produced by the hybridomas Springer is investigating. Although Mac-2 is strongly reactive with thioglycolate-stimulated macrophages, it is not so with resident cells of the peritoneum. No reactivity is noted with *Listeria*-activated macrophages, and only weak reactivity is seen with peptone-induced cells. A 32,000-dalton band, identified by this antibody, is expressed apparently preferentially on thioglycolate-stimulated cells. Mac-4 (54-2 of Russell) recognizes mast cells as well as macrophages and reacts with a 180,000-dalton molecule in the membrane. For comparison, anti-Fc recognizes a 47,000- to 70,000-dalton molecule on B-cells and macrophages according to Unkeless' results, and 2 distinct molecules of 52,000 and 67,000 daltons, respectively, with Fc binding properties have been isolated by Lane and associates. In man, Ortho-Kung monoclonal antibody and Mo 1 react similarly to Mac-1. Blood monocytes, granulocytes, and null, NK, and K cells, all react with these reagents, but Mo-2 fails to react with granulocytes. Mac-120 (Raff) stains cells involved in the production of cytokines and identifies 30% of blood monocytes but no granulocytes. In the discussion, Billing described antibodies of AML that reacted with 22 of 26 AML leukocyte specimens but none of 32 ALL specimens. A second monoclonal antibody reacted against ALL cells, and another acted only against blast cells and monocytes. Immunodiagnosis of the leukemias may be facilitated by such reagents.

Stollar presented data on his dissection of autoimmunity by monoclonal antibodies against altered self-components, with the model of SLE in the MRL-LPR mouse. A series of monoclonal antibodies against native or denatured DNA were studied, some of which were competitively inhibited by poly I or poly G, or both, but usually to different degrees. Some antibodies recognized the bases on DNA, and denatured DNA inhibited them well. Others recognized principally the backbone of DNA, and native DNA inhibited them. Recurring patterns in these categories were seen when a host of such monoclonal antibodies derived from hybridized lymphoid cells from the afflicted mice were examined. Anti-idiotype binding to the idiotypic site is competitively inhibited by denatured DNA, poly G, and poly I, which indicated a shared structure in the binding site. Cardiolipin (the Wasserman reagent) and phosphatidic acid inhibit several of the monoclonal antibodies. Homologies of these lipids with DNA phosphodiesters around the bases are thought to be the cause of this

inhibition. The 2 lipids also inhibit the binding of anti-idiotype antibodies to the idiotype, whereas antibodies against the bases rather than backbone components do not cross-react. Monoclonal H102 is the anti-idiotype described and is also an antinuclear antibody by fluorescence.

The treatment of leukemia by monoclonal antibodies in mice was described by Bernstein. The antibodies were directed against differentiation antigens, especially Thy 1.1 expressed on the AKR leukemia. Complement was not involved in the in vivo mode of action of the reagent. The IgG_{2a} antibodies were found most effective; IgM was inactive. Cell dependency of the antibodies (cytophilia, or arming capacity) was thought to be most important. Survival of the mice was prolonged by local excision of the lymphoma–leukemia and a single dose of antibody. Spontaneous leukemia was prevented by treatment of retired breeders with CPP or anti-Thy 1, or both. The median survival was slightly but statistically significantly better in mice given antibody, but 5 mice died from pneumonia without evidence of leukemia. Failure to cure the mice was partly due to selection of Thy 1-negative variant cells, which was not a modulation but a true loss of antigen-positive cells. Whether the T-cells of the host were adversely affected by the anti-Thy 1 antibodies was the subject of considerable discussion, but no conclusive affirmative evidence was given. This approach of investigators to the treatment of a tumor, i.e., an attempt to attack differentiation antigens rather than TAA, was hotly debated, and experiments such as Bernstein's will be closely scrutinized in the future for their selectivity of antitumor effectiveness.

Kaplan discussed his experience with human–human hybridomas, the first such hybridomas produced and of potentially profound importance, in work done in collaboration with Olsson. An IgE lambda myeloma U-266 was hybridized with various lymphoid cells, after selection of HGPRT-negative mutants that do not grow in hypoxanthine–aminopterin–thymidine medium and thus permit selection of hybridomas over parental myeloma cells. In two positive fusions, lymphocytes from patients sensitive to DNCB and thus reactive to the dinitrophenol backbone were hybridized. Five of 54 hybridomas produced specific anti-dinitrophenol antibodies of the IgG kappa type in one fusion. Antibodies reactive against other antigens, mainly of the IgM kappa type, have also been produced. Conditions for producing the human–human hybrids were described in detail, and it was noted that the HGPRT-negative U-266 line, now called SKO-007, is available to investigators upon request free of charge. A monoclonal antibody reacts against autologous Reed-Sternberg cells but not against normal cells from the same patient. Autologous hybridoma antibodies have a low titer (1:10), but there was no titer in the patients from whom the lymphocytes were obtained. In the discussion, the danger of enhancement of solid tumors through the use of antibodies was raised. Kaplan replied that he would use antibodies coupled to toxic molecules, such as ricin or alpha-emitting isotypes rather than rely solely on the antibody as a cytotoxic molecule.

Levy continued the discussion of antileukemia therapy, describing work he and Miller performed in patients. An antibody termed "17F12," equivalent to Leu-1, was used in these studies. It was reactive not only with leukemia cells but also with 90–95% of normal T-cells, reacting strongly to Sézary (T-helper) cells. This mouse anti-human antibody was from ascites but apparently was free of endotoxin, which is an important contaminant to avoid. Four patients were treated for several days to 10 weeks. Only 1 of the 4 developed antibodies to mouse Ig, which was treated by centrifugation at 100,000×*g* weekly to remove immunogenic microaggregates. Antigenic modulation occurred but was reversed within 2 days and was never a problem in therapy. In 1 patient, a temporary decrease in Leu-1-positive cells by 3–6 weeks after initiation of therapy was followed by a relapse at 11 weeks. The relapsing cells had antigen on them, even at a dose of 20 mg of antibody at that time. Whether one should use a mouse rather than a human antibody was discussed. Steplewski stated that despite the potential danger of C-type viral particles, he would prefer to receive mouse antibodies than any derived from a human myeloma cell. However, one can screen for human retroviruses; C-type viruses are not found in human myeloma in any event.

Nude mice bearing a human tumor were treated by Royston, Dillman, and their collaborators. Radionuclides to the antibodies were found, mainly against CEA, and their pharmacology was determined. Rapid urinary excretion was a problem with [^{125}I]-labeled material but less so with indium-111-labeled antibodies. Localization with indium-111 was severely limited by uptake in the RES, probably after formation of immune complexes. Human phase II trials have been started with the use of an infusion of (unlabeled) antibody during a 15-minute period. No circulating antigens were detected. Problems associated with administration of the antibodies were emphasized here, in contrast to Levy's presentation, because an anaphylactoid reaction occurred at a dose of 12 mg of antibody. Urticaria, cough, dyspnea, diarrhea, and hypotension were distinct problems. Macroaggregates could not be detected, but they were suspected because dyspnea and hypotension occurred after the first dose of 10 mg rather than after a series of injections. Slow infusion may be less toxic.

Dippold discussed the work at the Sloan-Kettering Cancer Center and elsewhere on antimelanoma antibodies, in which a wide range of antigenic protein, glycolipid, or carbohydrate determinants were analyzed as possible critical molecules detected by the antibodies. A whole series of proteins have been identified by monoclonal antibodies by several research groups, including HLA, beta-microglobulin, DR, and other more specific proteins and glycoproteins. Three glycolipids and one unspecified molecule from the Wistar laboratory group (19-19) have also been found. A ganglioside, R_{24}, is found on melanomas, normal melanocytes, and, to some degree, on brain cells, but otherwise it is not on normal cells. Melanoma-specific antigens seem to be detected on at least some of the melanomas studied. In the discussion, Reisfeld cautioned that the M14 melanoma and the T24 bladder tumor share a 94,000-dalton antigen with a similar isolectric point, which is probably identical with the P97 antigen identified by Dippold et al. and may be an oncofetal antigen rather than a melanoma-specific molecule. On the other hand, a

240,000-dalton protein associated with the membrane is highly specific. Standards are essential if we are to judge accurately the molecular weight of some of these molecules, particularly for glycoproteins for which polyacrylamide is a poor medium for such determinations. The 240,000-dalton molecule may be a good candidate for the localization of melanoma in normal viscera. Hellström and Brown obtained antibodies to gp95 (pg97) and have quantitated the amount of antigen on various cells. Only melanomas have the antigen; normal gut and bone marrow lack it. Antibodies to 2 epitopes killed in the presence of complement, but only when they were combined. Tumor imaging in vivo with as little as 2 μg of antibody and 1 mCi of technetium-99 has given encouraging results thus far. Ferrone indicated that at least 4 antigens are in the p94 range and urged caution at referring to a single antigen of that weight. Normal melanocytes lacked the antigens seen by anti-p94 as well as a cell membrane component identified by an antibody known as 230. Nevi had antigens reacting with both p94 and 230 but not Ia-like (DR) molecules, whereas melanoma cells had all three specificities. Thus distinct patterns that identify malignant cells are emerging from several investigators, even though absolute tumor-specific antigens have remained elusive.

Steplewski described his group's results with anticolorectal tumor monoclonal antibodies. The main difficulty they encountered was that some antibodies seemingly specific for colon tumor antigens cross-react with minor blood group antigens (such as the Lewis) or with meconium. Nevertheless, their applicability to many diagnostic and therapeutic situations may be undiminished. A monoclonal antibody, called 19-9, reacts against colon carcinoma and meconium and helps in the detection of a monosialoganglioside confined to the surface of the tumor. Patients with pancreatic carcinoma also had the antigen. This antigen is not present in normal intestine nor on RBC but is found in the sera of cancer patients. Patients with inflammatory bowel diseases or smokers do not have detectable antigen. No correlation was observed between the presence of the monosialoganglioside and CEA. Monoclonal antibodies to colon carcinoma can inhibit the growth of tumors in nude mice and are active only against the colon cancer, not against breast or lung carcinomas. Whether the antibody acts to kill the tumor by arming effector cells is uncertain; although it is active in ADCC in vitro, $F(ab')_2$ fragments are also effective in vivo. Steplewski also described antibodies to human melanoma that detect antigens common to all melanomas but do not bind to normal melanocytes in the skin. Nevi in culture express the antigens after several passages, and the antibody also reacts with astrocytomas. Additional interesting monoclonal antibodies to various human cancers were described, notably antipancreatic antibodies (Metzgar R), anti-oat cell carcinoma of the lung antibodies (Minna J), and antileiomyosarcoma (Billing R).

Byrd reported that a monoclonal antibody reacting against all 6 lines of neuroblastoma tested also reacted against fetal but not adult brain. Interestingly, no reactivity was seen in other fetal tissues. The antibody detected single cells in the bone marrow by the immunoperoxidase technique and greatly enhanced the sensitivity of detection of metastatic neuroblastoma. Thirty-two of 34 patients at diagnosis had bone marrow involvement by this assay, and 11 of 11 at relapse. Nineteen of 26 assumed to be in remission had an ostensibly positive bone marrow, yet when leukemia cells were sought with the anti-neuroblastoma antibody in patients with ALL, 44% of the patients in presumed complete remission had leukemia cells in the marrow. The antibody reacted against B-ALL cells and non-T, non-B-ALL. Thus immature lymphocytes in the bone marrow of neuroblastoma patients might have been detected by the antibody in some instances rather than neuroblastoma cells. To test this, Byrd assayed an antibody that reacted only against lymphocytes (not against neuroblastoma cells) against the bone marrow with the first monoclonal antibody. Ten of 15 patients with disseminated neuroblastoma had reactivity with the first but not the second antibody, whereas about 50% of the 99 patients in remission or at presentation had reactivity to both. By double labeling with the 2 antibodies, one can obviate the need for a single tumor-specific antibody to detect neuroblastoma without excessive false positivity. This is obviously a principle that can be applied to many other situations if one has antibodies with complementary specificities. Antibodies to neuroblastoma inhibited the growth of neuroblastoma cells in tissue culture, in the absence of effector cells, which suggested a direct effect on the metabolism of the tumor cells.

During the discussion, Seeger described an antibody to neuroblastoma cells that recognized Thy 1 derived by immunization with fetal brain. Thy 1 was found on a host of tissues, including rhabomyosarcomas, leiomyosarcomas, gliomas, teratomas, and an occasional Wilms' tumor. The normal counterparts of these tumors of embryonic origin also had the antigen. Considerable heterogeneity of this antigen was expressed within a neuroblastoma, but no differences in expression were observed between local and metastatic sites.

Solter has shown that monoclonal antibodies can be used for detection of tumor by radiolabeling them with conventional radionuclides, such as iodine-131. An antibody to a mouse teratocarcinoma detected stem cells in the embryo at the 8-cell stage and beyond. A neutral glycolipid is the critical determinant and is related to the blood group antigens in the human H(O) group, especially antigen "I." This antibody detects a structure that is apparently rare in the mouse but is found in human tumors, such as colorectal carcinoma and choriocarcinoma, but not in human teratocarcinoma. Granulocytes and myeloid lines also express the antigen. Inasmuch as the structure of the relevant antigen is known, it is now possible to investigate the genetic control of its synthesis from its primordial globoside precursor. A second monoclonal antibody detects antigens present in unfertilized eggs to the morula stage in the mouse. Nine of 11 teratocarcinomas but not myeloid lines react with this antibody. Cord and adult RBC do not react. The antibody, in contrast to the first, detects a carbohydrate determinant. Scientists conducting radiodetection studies have used the first antibody, known as SSEA-1, labeled with iodine-131. The concentration of antibody is fivefold to tenfold greater at the sites of tumor than in normal tissues. The $F(ab')_2$ fragments are superior

to whole antibody, even though they are initially excreted more rapidly because the second phase of clearance is much slower than that of whole antibody and especially because nonspecific binding through the Fc portion is eliminated. Even with whole antibody, nonspecific localization can be controlled with the use of a [^{123}I]-labeled unrelated antibody and subtraction of its activity as background. Twenty to 50 mg of tumor can be detected by this monoclonal antibody, with no nonspecific binding in the kidney.

Kaplan, in an invited summary, pointed out some future directions of the field and suggested that dissection of the immune response will be greatly facilitated. At the clinical level, bacterial and fungal disease due to toxins can be treated by highly specific human IgG. Such occult carcinomas as pancreatic cancer can be detected early and their course followed carefully by antibodies that react with circulating antigens or are used for radiolabeling of the tumor in situ. Surgical pathology, so long reliant upon standard histology, can be advanced by the use of highly specific monoclonal antibodies as diagnostic reagents, which can be used even on fixed sections through the immunoperoxidase techniques. Several important problems are now evident from administration of the antibodies to patients. Careful phase I trials of antitumor antibodies are necessary because some toxicity has been described, including sudden death in a few patients. The degree of stripping of the radionuclide label, or of a toxin, from the monoclonal carrier antibody must be measured accurately in phase I trials too. One could use radiolabeled intralymphatic monoclonal antibodies to detect melanoma in regional lymph nodes for prognostic purposes, an improvement on the radionuclide lymphangiogram. Kaplan personally prefers an alpha-emitting agent as a toxic tail to a specific antitumor antibody, but many substances, including ricin or diphtheria toxin, could be used instead. Such heterogeneity of expression of antigens may occur in a single tumor that a panel of antibodies might be needed for diagnosis or treatment. Relapses might occur when antigen-negative cells are present that do not express the first antigen detected, thereby requiring a different antibody. Sanctuaries in which relapses occur might also require a different route of administration of the antibodies. If tumors are to be treated with antibody, those that kill the host most rapidly should be chosen as subjects, so that one can see the beneficial effects of antibody most quickly. Finally, another application might be that one rid the bone marrow of tumor cells before reinfusion into a patient heavily treated with chemotherapy or radiation. This could provide to normal bone marrow what would otherwise be lethally marrow-toxic therapy.

The participants in the workshop were unanimously enthusiastic but by no means sanguine about the value of monoclonal antibodies, the enormous potential of which must now be harnessed through careful identification of their target epitopes and range of reactivity, pharmacology in vivo, and their toxicity to the host before proceeding much further.

WORKSHOP ON LYMPHOKINES AND THYMIC HORMONES AND THEIR POTENTIAL UTILIZATION IN CANCER THERAPEUTICS

Recent advances in immunology and immunopharmacology indicate that lymphokines and thymic hormones are important in increasing host immunity and in modifying biological responses in patients with cancer and other diseases associated with immune dysfunction. Because of the rapid expansion of scientific knowledge of these natural substances, the BRM Subcommittee of the Board of Scientific Advisors of the Division of Cancer Treatment, NCI, organized a workshop during which participants would explore the potential utilization of lymphokines and thymic factors in cancer therapeutics.

Experts, representing a cross-section of the fields of lymphokines and thymic hormone research, were invited to participate in a workshop to review recent developments in the chemical characterization, mechanisms of action, regulation, and potential utilization of these BRM in cancer therapeutics. They were asked to analyze critically the state of the art, including the intracellular mechanisms controlling lymphokine and thymic hormone action in biological response modification. They were also asked to address the potential clinical applications of lymphokines and thymic hormones in cancer research therapy.

The workshop was divided into 3 major categories. The first dealt with the purification and characterization of lymphokines and lymphokine stimulators. The state of the art used to define the biochemical characterization of the interleukins, lymphotoxins, TF, and other T-cell growth and proliferation factors as well as lymphokines that stimulate nonlymphoid cells was reviewed. In addition, a description of monoclonal antibodies against several of the lymphokines and detailed methods for the purification of several of the lymphokines and detailed methods for the purification of several of the biologically active molecules were discussed.

The second part of the meeting was devoted to biological and molecular models of lymphokine and thymic hormone function. In this section, the function of these agents in modulating lymphocyte and monocyte populations was explored in depth, including mechanisms of cell–cell communication, pharmacological modulation, and regulatory properties. The roles of several of the suppressor lymphokines were discussed, and data outlining their biological properties in vivo and in vitro were presented.

A third part of the meeting was devoted to the potential role and current status of lymphokines and thymic factors in cancer therapy in humans and animals. Immunotherapeutic effects of thymosin and lymphokines were reported in a number of tumor models in animals, including the use of liposomes to achieve activation of tumoricidal properties of murine macrophages. The final part of this session reviewed the status and potential use of lymphokines in the treatment of advanced cancer patients.

DeWeck gave the keynote address and discussed the biological roles of lymphokines, monokines, and cytokines. These activities included: the maturation and differentiation of lymphoid cells; mobilization of lymphoid and inflammatory cells; intercellular cooperation; regulation of

systems; activation of lymphoid and nonlymphoid cells, including inflammation, growth, regeneration, and the killing and inactivation of target cells.

He also summarized the major problems in the field which, until recently, limited progress. These include: 1) Lymphokines are usually produced by a mixture of cell types and may often require cooperation (e.g., among macrophages and lymphocytes) for effective production. 2) Correspondingly, any culture supernatant from activated cells usually contains several lymphokines, monokines, and cytokines. 3) The target cell population used for assessing the biological activity of lymphokines is itself frequently heterogeneous and capable of producing them and monokines as well as other factors having an enhancing or inhibiting effect on the biological assay under study. Under such circumstances, investigators' attempts to ascribe molecular identity to factors exhibiting different biological activities in vitro when dealing with the "black box" of crude culture supernatants have amounts to little more than wishful thinking. 4) Chemically characterized molecules are lacking.

DeWeck discussed the prospects for utilizing lymphokines as therapeutic agents. He pointed out that because so many of the cells and mechanisms involved in tumor defenses are directly produced and affected by lymphokines, the need is dictated for a continued evaluation of their role and use in cancer therapy. He also cautioned that their actions have many facets and that these substances should not be used indiscriminately. In particular, the negative feedback loops present in all the immune circuits must be carefully evaluated. For example, lymphokines have recently been shown to raise steroid levels in mice and in humans and appear to influence IL-1 production and activity.

Furthermore, he indicated that the key areas for continued research should include: standardization of biological assays for lymphokines; large-scale production of several human lymphokines; and production of monoclonal antibodies against the lymphokines to help in their identification and quantitative evaluation in pathological conditions, structural characterization and mass-scale production, and evaluation of their functions in vivo and selective access to immunological regulation.

Mizel reported that considerable progress has been made in purification of murine III from a macrophage cell line ($P388P_1E$) with a superinduction technique. He reported that when $P388D_1$ cells were incubated with PMA (10 μg/ml) for 5 hours, washed, and incubated for an additional 24 hours, little if any supernatant II was detected. In contrast, incubation of the cells with cycloheximide and PMA for 4 hours, followed by a 1-hour incubation with dactinomycin, resulted in the production of approximately 900 U/ml of IL-1. This value can be compared with 11 U of IL-1 produced when $P388D_1$ cells are incubated with PMA for 6 days. He also reported that the production of IL-1 can be further enhanced if cells are also supplemented with sodium butyrate throughout the entire incubation.

Using this technique, Mizel detected as much as 1–2 mg of IL-1/liter of superinduced $P388D_1$ cell culture fluid. The results of his study indicate that sufficient quantities of murine IL-1 can now be obtained for biochemical and biological characterization studies.

Lackman spoke on the purification to homogeneity of a human IL-1 from leukemia cells from AML culture medium. He found that leukemia cells 1) offer a valuable source of IL-1 and other factors because the cells can often be obtained in great quantity (10^{11}–10^{12}) when patients require therapeutic leukophoresis; 2) can be frozen or used at once to prepare large quantities of IL-1 from a *single* donor; and 3) can be cultured at high densities to result in an extremely active conditioned medium.

He also reported the partial purification of a human IL-2 using Ficoll-Hypaque-purified peripheral blood cells stimulated with PHA and an allogeneic human B-cell line. Lackman noted that purification of human IL-2 progressed slowly due to the lack of a cell source which releases large amounts of activity and IL-2 exhibited biochemical and biological heterogeneity not exhibited by IL-1.

A human TCGF initially described by Gallo and his group in 1976 has not been purified to apparent homogeneity from serum-free lymphocyte-conditioned media by utilizing ion exchange chromatography with DEAE–Sepharose, gel filtration with Ultragel AcA54, and preparative SDS–PAGE. This mitogenic protein has a mol wt of 13,000, as determined by SDS–PAGE and 20,000 to 25,000 by gel filtration, and an isoelectric point of 6.8. The material extracted from acrylamide gels is a single homogeneous band when analyzed on analytical SDS gels. The highly purified material is unstable at −70° C and requires the addition of BSA or polyethylene glycol to maintain biological activity. It is sensitive to proteolytic digestion but resistant to nucleases and thiol reducing agents such as dithiothreitol. The purified factor has sustained T-lymphoblasts in tissue culture and lacks other lymphokine activities, including CSA, IF, and B-cell mitogenic activity. Gallo also reported that, in contrast to lectins, such as PHA, certain antigens, and crude lymphocyte-conditioned media, purified TCGF does not initiate lymphocyte blastogenesis but is a highly selective mitogen for T-cells previously activated by exposure to lectins or antigens. His group is currently conducting amino acid sequence analyses of this purified molecule.

Farrar, in a progress report on the development of larger amounts of murine IL-2, indicated that he used PMA-stimulated EL 4 thymoma cells as a source of large quantities of IL-2. Through the use of sequential ammonium sulfate precipitation, phenyl Sepharose chromatography, Tris glycine PAGE, and two-dimensional electrophoresis (isoelectric focusing followed by SDS-gel electrophoresis), he purified IL-2 to approximately 4,000,000 U/mg protein. He is proceeding with work on final purification of the molecule.

Gillis related the success he and his co-workers had in purifying a murine and human IL-2 to homogeneity using conditioned media from several leukemia and lymphoma cell lines. A human IL-2 with a mol wt of 14,000 purified from a leukemic T-cell line (Jurkat-FHCRC) is currently being sequenced. A single N-terminal amino acid analysis was reported to confirm homogeneity. Gillis also reported the development of monoclonal antibodies for these molecules which recognize determinants present on IL-2.

In his presentation, Stadler reported that he and his associates also were successful in producing monoclonal antibodies against a human IL-1 (obtained from culture supernatants of human mononuclear cells stimulated with LPS and human IL-2 that was obtained from cells stimulated with Con A and PMA in the presence of hydroxyurea). He indicated that these monoclonal antibodies should provide novel tools for the study of the regulatory role of these mediators.

Ihle described the purification and biological properties of an interesting lymphokine which has been termed IL-3. This molecule apparently promotes an early step in T-cell differentiation. It has a mol wt of approximately 40,000 and is a potent inducer of 20α-steroid dehydrogenase, an enzyme marker found in hydrocortisone-resistant T-cells in the thymus and in most peripheral T-cells. Sequence analysis of this protein is underway. With regard to the physiological significance of IL-3, Ihle indicated that, although considerable work is necessary for better delineation of the precise physiological role of IL-3, the available data provide some interesting speculative possibilities. For example, the fact that IL-3 promotes differentiation of early T-cells suggests that, with regard to amplifying the immune system in vivo, IL-3 has more general effects than factors, such as IL-2, which amplify terminal components of the system. Also, inasmuch as IL-3 appears to be the initial limiting factor with regard to differentiation of T-cells in nu/nu mice, replacement therapy in syndromes such as DiGeorge's disease and other T-cell hypoplasias may be possible. Because IL-3 can facilitate the differentiation of functional helper T-cells, it may be useful as an immune adjuvant to facilitate the initial recruitment of helper T-cells.

Granger discussed the continuation of his studies on the purification and properties of a complex system of interrelated cell toxins, termed "lymphotoxins," produced from iodinated lymphocytes. The complexity of these molecules is approaching that of the complement system. His evidence indicated that some of the lymphokines may be derived from a common precursor. He also introduced the prospects for therapeutic utilization of the lymphotoxins. Reports from many investigators lend support to the concept that lymphotoxins may be selectively cytotoxic or cytostatic for transformed cells compared with normal cells in vitro. Evidence is available that these molecules are tumoricidal in vivo in both humans and animals. Whereas these observations are exciting and potentially fundamental, Granger correctly pointed out that they are severely limited by the fact that extremely crude lymphocyte supernatants were used as lymphotoxin preparations in many of the studies. Because these supernatants contain multiple lymphokine activities with anticellular effects, it is not clear that lymphotoxin is the only causative agent. Even those investigators who attempted to control for resident IF, e.g., cannot readily discount the possibility of synergy between different anticellular mediators (as has been observed for different IF).

McEntire described the isolation and purification from a human lymphoblastoid cell line of a novel acid-soluble polypeptide with potent macrophage phagocytic promotion and activation. Of major potential clinical significance is the potent antitumor activity of this partially purified lymphokine preparation. The active peptide(s) was reported to have an apparent mol wt of 8,400 and to consist of approximately 0.5% aspartic and glutamic acids.

The session on the chemical characterization of lymphokines was completed by a presentation by Burger on human TF. Burger's studies argue for the concept of a specific TF raised through immunization and support the specificity of TF reported in the literature. His results indicate that the TF structure possesses a polypeptide component with a free carboxy terminus and a phosphodiester linkage to a moiety with a free 3′-hydroxyl. Retention characteristics on high pressure, reverse-phase chromatography, and behavior to alkaline phosphatase treatment are consistent with an additional phosphate residue in the TF molecule.

The next section of the meeting dealt with the biological and molecular models of lymphokine function. Sorg described his studies on the response of macrophages to MIF and other lymphokine signals. Some of the important studies he and his colleagues have underway include those on the chemical functional characterization of MIF in 3 species (man, guinea pig, and mouse) and others dealing with the analysis of the mononuclear phagocyte system and its response to MIF and other cell signals. He and his coworkers have now purified from Con A-stimulated spleen cells, molecules exhibiting MIF-like activity with mol wt of 14,000, 28,000, 42,000, and 56,000. Molecules having mol wt of 28,000 and 56,000 showed biological activity, whereas only minor activity was observed in the others. On isoelectric focusing, MIF-like activity was found for all molecular weight ranges at pH of 4.5–5.5. Neuraminidase treatment did not destroy activity. These properties are similar to the chemical properties for guinea pig MIF and suggest that the inhibitory activity is associated with a group of structurally similar molecules rather than a single molecule. Sorg also reported that chemical characterization on human MIF is proceeding and that they have similarly identified several molecular species ranging from 10,000 to greater than 60,000 daltons. The isoelectric points of the various species is approximately 5.2.

Sorg's presentation also included an interesting hypothesis on the biological basis of macrophage heterogeneity. According to his proposed model, one can divide lymphokines and also cytokines into differentiating and activating signals. Until now, only cumulative effects on macrophage function with a cocktail of lymphokines have been studied. With the use of purified lymphokines in macrophage populations that are functionally characterized, he proposed that it should be possible to define the precise nature of a lymphokine signal and the nature of the cellular response to it. Although it is still widely believed that MIF and MAF are different manifestations of the same molecule, we now have evidence that they are not identical. The basis of Sorg's model would differentiate among responses of various lymphokines in G_0, early G_1, late G_1, and other parts of the cell cycle. He proposes that differentiation, activation, and homeostasis of the mononuclear phagocytic system is regulated by a complex network of lymphokines and other factors.

Thurman presented important new information dealing

with the role of thymic hormones on the induction of specific and nonspecific macrophage migration inhibition. Previously, Thurman and his colleagues reported that the loss of reactivity to PPD after guinea pigs were thymectomized could be demonstrated by using the capillary tube macrophage migration inhibition technique. They showed that the responsivity of the PBL to PPD could be restored in this assay by adding in vitro either thymosin fraction 5 or a purified thymic hormone termed thymosin α_1. Thurman presented new information indicating that 2 newly isolated and sequenced thymosin polypeptides, thymosin β_3 and β_4, have the capacity to inhibit macrophage migration directly, independent of antigen. Their studies demonstrate that thymosin β_3 and β_4 act directly on macrophages. He proposed that thymosin β_4 may be the active component of the MIF molecule itself, or it may have a common homology with the active site of MIF. Now that a thymic peptide that significantly inhibits the migration of macrophages has been sequenced and synthesized, its availability for study should help in further delineation of the structure and function of MIF.

Hadden discussed lymphokine-induced macrophage proliferation and activation. Macrophage proliferation and activation induced by T-lymphocytes is mediated by lymphokines. A number of factors produced by sensitized thymic-dependent lymphocytes have been shown to act on macrophages. These include: 1) A macrophage growth factor previously termed "macrophage mitogenic factor," which induced mature nonsensitized macrophages to replicate; 2) an MIF which inhibits macrophage migration; 3) a macrophage aggregating factor which agglutinates macrophages; 4) an MAF which stimulates protein synthesis and hexose monophosphate shunt activity in macrophages and increases listericidal and tumor cell killing activity of macrophages; 5) a macrophage chemotactic factor which induced macrophage chemotaxis; 6) CSF which regulate the proliferation and differentiation of granulocytes and macrophages from a precursor colony-forming cell; 7) "immune" IF (γ-IF) which inhibits viral replication and may activate macrophages to kill tumor cells; and 8) a macrophage fusion factor which induces giant cell formation.

Hadden suggested that the macrophage growth factor is a logical candidate to be used immunotherapeutically to enhance the expression of DTH reactions to cancer or pathogens, or both. He indicated that in cancer therapy, this lymphokine might be used alone to increase macrophage numbers, or in conjunction with other lymphokines or immunostimulating agents to promote macrophage activation for tumoricidal activity.

McDaniel reported on the further chemical characterization of MAF produced by Namalva cell lines, a human Burkitt's lymphoma. She noted the presence of multiple components with mol wt of 12,500, 25,000, 70,000, and 1 fraction of less than 10,000. Using comparable chemical fractionation procedures, she isolated human MAF from tonsil lymphocytes and found that its activity was associated with a 12,000-dalton fraction. McDaniel's studies indicate that MAF consists of multiple units which form from a low molecular weight unit by some as yet unknown mechanism of association.

The pharmacological modulation of the interaction between lymphokines and macrophages, particularly the effect of antioxidants and thromboxane synthetase inhibitors, was presented by Pick. Molecular mechanisms by which lymphokines affect the target cells are by and large unknown. Thus lymphokine action is usually expressed as biological activity, which frequently can be the expression of multiple biochemical processes. Efforts by Pick and his associates led to the elucidation of a possible mechanism of action of MIF on macrophages. He proposed a working model for MIF action that involves the participation of unidentified oxygen radicals and thromboxane. This model is summarized as follows:

Sequential biochemical events in the activation of macrophages by MIF

- Early immobilization following chemotaxis (0–2 hr)
  - Direct effect of oxidative burst products (O_2, H_2O_2, OH, or O_x) on locomotory apparatus of cell membrane
  - Increase of cell adherence and aggregation by stimulation of thromboxane A_2 synthesis
  - Fall in cAMP (prostaglandin endoperoxide-, thromboxane-, or H_2O_2-mediated?)
- Transition stage (consolidation of immobilization, shift to activation for 2–24 hr)
  - Increase in tubulin polymerization and microtubule generation
  - Enhanced actin polymerization
- Activation stage possibly mediated by a distinct lymphokine (24–72 hr)
  - Enhanced propensity for oxidative burst upon membrane stimulation but autotoxicity prevented by:
    - Hexose monophosphate shunt stimulation linked to enhanced glutathione peroxidase activity
    - Elevated catalase level
  - Thromboxane synthesis replaced by PGE_2 production
  - Enhanced secretory function (neutral proteases, acid hydrolases, complement components, etc.)

Pick proposed that the cessation of cell movement, induced by oxygen radicals and thromboxane, results from the effect of oxygen radicals principally on the locomotory apparatus itself, whereas thromboxane affects mostly membrane properties such as cell adhesion.

A report on the chemical characterization of antigen-specific suppressor lymphokines was given by Webb. Of the suppressor factors isolated to date, some are specific to the class of antibody which is regulated, the type of regulated response (e.g., antibody vs. DTH), and for the given type of determinants (e.g., carrier vs. hapten specific). Most recently, several scientists reported a preparation of T-cell hybrids which constitutively produce some of these suppressor factors. The development of hybridomas should facilitate the purification and analysis of some of these suppressor factors.

Pierce discussed the structure and mechanism of action of the lymphokine-soluble immune response suppressor, a product of Con-A-activated murine Lyt 2^+ T-cells that nonspecifically suppresses immune responses in vitro. After exposure to this suppressor, macrophages release a macrophage-derived suppressor factor, which is a potent inhibitor of proliferation of T- and B-cells and several

tumor cell lines. Effects of this macrophage-derived suppressor factor on cell division are rapid but reversible. Several observations suggest that this factor may be an oxidizing agent. The macrophage-derived and soluble immune reponse suppressor factors are proteins with mol wt of about 55,000 and have similar properties. They suggest that the latter factor is a peroxidase converted by macrophages or H_2O_2 to this macrophage factor which is an oxidizing agent with biological activity that apparently is mediated by oxidation of cellular components essential for cell division.

Cianciolo's investigations on the characterization of inhibitors of monocyte function present in effusions of cancer patients are based on the fact that the tumor itself made locally or systemically abrogates the effects of immunotherapeutic approaches by producing molecules that will suppress monocyte functions. The observation that surgical removal of the tumor usually results in normalization of patients' monocyte chemotactic responses suggests that the tumor itself is responsible for the defect in monocyte function in tumor-bearing individuals, perhaps by the release of a soluble mediator. His studies reveal the presence of a soluble mediator with a mol wt less than 25,000 that is relatively heat stable and trypsin sensitive. Monoclonal antibodies reactive to the P_{15} (E) component of type C retroviruses are capable of absorbing the inhibitory activity from cancerous effusions. Use of an immunoabsorbant column prepared with monoclonal anti-P_{15} (E) resulted in isolation of inhibitory material with a greater than 100,000-fold increase in specific activity.

The in vitro studies on macromolecular synthesis in lymphokine-producing and responding cells by Varesio and his colleagues indicate that, upon activation, lymphocytes undergo a complex series of changes which lead to the acquisition of many immunologically relevant activities such as proliferation, cytotoxicity, antibody synthesis, and lymphokine production. The production of lymphokines is one of the earliest events associated with the activation of lymphocytes, by either mitogens or antigens, with lymphokines becoming detectable in culture supernatants within 46 hours after stimulation. Varesio found that, with treatment of lymphocytes with either mitogens or antigens, an early step dependent on protein synthesis is absolutely required for activation. This key step seems to be one of the targets for a regulatory signal from suppressor macrophages because they can inhibit the protein synthesis of lymphocytes during this early critical time after stimulation. He postulates that activated macrophages suppress lymphocyte functions through an inhibition of lymphocyte protein synthesis. The studies are supported by the observation that various immune functions which require protein synthesis are sensitive to macrophage-dependent suppression, whereas functions independent of protein synthesis, such as T-cell-dependent cytotoxicity, are not.

The in vivo development and regulation of cytolytic T-cells specific for altered self-antigens were discussed by Battisto, who, with his colleagues, described a method for inducing cytolytic T-cells in vivo to hapten-altered syngeneic cells. They used this new method to examine the development and regulation of this form of CMI. The method consists of stimulating the hosts helper T-cells with a disparate auxiliary cell at the same time that hapten-altered syngeneic spleen cells trigger precursor cytolytic cells.

The last presentation in this section of the meeting was on the utilization of immune IF as an antitumor agent. Baron reviewed this area in a discussion of data indicating that immune IF manifests many major differences in comparison with fibroepithelial and leukocyte IF. These differences make immune IF potentially attractive as a possible anticancer agent. He pointed out that immune IF, both in the crude and more highly purified states, exerted a greater antitumor action relative to its antiviral action than other types. He also reported that the anticellular action of immune IF is ten to fifteen times greater than that of the other two types. The differences between immune and other types of IF include a greater anticellular effect and potentiation of effects on immunity. These activities make immune IF particularly attractive as an antitumor agent for eventual application clinically.

The third part of the meeting was devoted to the potential role and current status of lymphokines and thymic hormones in cancer therapy in humans and animals. Numerous important immunotherapeutic effects of thymosin and lymphokines were reported, and several models in animals were described, including the use of liposomes for activation of tumoricidal properties of murine macrophages. This section of the meeting also included a review of the status and potential use of lymphokines in therapy of advanced cancer.

The effects of combined CPP and thymosin treatment on tumor growth and host survival in mice bearing syngeneic tumor was presented by Zata. In her study, administration of thymosin fraction 5 in multiple microgram doses over a 2-week period following CPP treatment resulted in long-term survival of BALB/c mice bearing the syngeneic MOPC-315 plasmacytoma. The dose of thymosin fraction 5, as well as a dose of CPP, was critical in the prevention of recurrence. Her studies indicated that thymosin fraction 5 was effective in prolonging survival in tumor-bearing animals following chemotherapy. Thus we have an important animal model for further study of thymic hormones in immunotherapy of cancer.

In an update on his ongoing thymosin trial in patients with head and neck cancer following radiotherapy, Wara reported that local irradiation (5,000 to 6,000 rad) produced a secondary suppression of the immune response. His results indicated that thymosin-treated patients recovered their immune responsivity early and had a longer relapse-free interval. He observed that, because of the positive effects of thymosin, further investigations in patients with head and neck cancer and other forms of cancer are necessary to determine the optimal dose of thymosin that will yield the greatest biological effects.

Fidler discussed the use of liposomes as carriers for lymphokines to achieve activation of tumoricidal properties in murine macrophages and destruction of pulmonary metastasis. His data revealed that in situ activation of tumoricidal properties in murine macrophages was possible following the iv administration of liposomes containing lymphokines. This approach was effective in the treatment of spontaneous pulmonary metastases in mice.

In the in vivo biological studies with lymphoblastoid lymphokines conducted by Papermaster and co-workers, this lymphokine preparation was nontoxic in humans; however, it was a potent antitumor agent in mice bearing the L1210 lymphoma. According to the results of their studies, lymphokines are immunostimulatory and may have a potential adjuvant role with chemotherapy in the treatment of neoplasms.

The in vivo release of lymphokines in relationship to CMI was studied by Salvin and his colleagues. They determined that lymphokines can be induced to be released in vivo by any one of several antigens and that the capacity to form MIF and to develop cell-mediated resistance are correlated. Salvin also indicated that the capacity of a given strain of mice to release MIF or IF did not correlate necessarily with the capacity of that strain to release other lymphokines into the circulation.

Neta discussed in vivo effects of immune IF in relationship to other lymphokines and the effects on the growth of tumor cells. Treatment with a lymphokine preparation containing 300 U of γ-IF had a significant effect on tumor growth which was dose dependent. Lower doses resulted in inhibition of growth of tumors, whereas high doses caused elimination of tumors. She also reported that γ-IF was much more effective than Type I IF in suppressing tumor growth; also, inhibitors to γ-IF may be in sera of low responder strains.

The short- and long-term effects of administration of a lymphoblastoid cell line lymphokine to patients with advanced cancer was presented by Dumonde. In his study, this lymphokine was isolated from supernatants of human lymphoblastoid cultures and, after partial purification, was given to patients with carcinomas of the breast and prostate and to 1 patient with Ewing's sarcoma. This clinical study indicated that lymphokines, administered locally and systemically, were well tolerated. No significant short- or long-term toxicity over a 2-year period of systemic administration was noted. Furthermore, the effects of the lymphokine from the lymphoblastoid cell line seem to relate to the host defense mechanisms, yet the IF system was not involved; also, long-term administration of a partially purified preparation to cancer patients was without significant toxicity. Dumonde's study provides important biological response data on which one can base more definitive protocols for the evaluation of lymphokines in cancer research therapy in the future.

It was apparent from the studies presented at the conference that great strides and progress are being made in our understanding of the chemical nature of several of the thymic hormomes, such as thymosin α_1 and thymosin β_4, as well as in the purification and characterization of several of the important lymphokines, such as TCGF, IL-2, and the lymphotoxins. The chemical and biological activities of a new lymphokine termed IL-3 were presented for the first time. The basic and clinical research efforts presented revealed that biologically active molecules, collectively designated as lymphokines and thymic hormones, have essential roles in the regulation of the immune system and in antitumor defense mechanisms. It also becomes apparent that much of the research with lymphokines has been done with crude preparations and a major need exists for further chemical characterization of the biological factors.

The availability of several of the purified lymphokines and thymic hormones for the utilization of large-scale production techniques, chemical syntheses, and recombinant DNA cloning techniques is beginning to provide researchers with enough of these important biologically active agents to study in the laboratory and clinical settings. The results of all these dramatic developments will hopefully provide novel approaches to the treatment of cancer in years to come. It was apparent from the presentations that lymphokines and thymic hormones are important BRM and further research into their chemical and biological properties should provide us with new approaches to the treatment of cancer and other diseases associated with immune imbalances.

WORKSHOP ON THE POTENTIAL ROLE OF T-CELL SUBPOPULATIONS IN CANCER THERAPY

This workshop brought together scientists studying the interaction between T-cell subsets in immunity, the generation and maintenance of reactive T-cells in vitro, and the positive or negative effects of T-cells on tumor immunity or therapy in vivo. Their goal was to assess current research results, identify some of the major areas which require increased investigative attention, and to begin to identify some of the problems which must be resolved to facilitate the eventual recognition of the optimal role for T-cells in cancer therapy.

In an overview of lymphocyte transfer as potential cancer therapy, Fefer emphasized that this area is now receiving far greater investigative attention largely stimulated by 1) an increasing number of animal models in which adoptively transferred T-cells have been therapeutically effective against advanced tumors; 2) the recently acquired ability to generate in vitro animal or human T-cells with antitumor reactivity and maintain in culture in the presence of IL-2; 3) the increasing knowledge about T-cell subsets and their interactions in the immunological network in man and animals; and 4) the apparent graft-versus-leukemia effect observed in patients receiving an allogeneic bone marrow transplant.

Shu studied tumor immunity in vivo and in vitro in a syngeneic guinea pig hepatoma model using 2 tumor lines, each with demonstrable non-cross-reactive TSA. Adoptive immunity, as measured by the growth of tumor cells inoculated shortly after infusion of spleen cells from immunized donors, was specific and quantitative. The immune cells were not specifically attracted to the site of tumor challenge. By contrast to effectors of adoptive immunity, in vitro cytotoxicity with a 48-hour [^{3}H]-release assay was not specific and the cells which transferred immunity in vivo had no demonstrable specific in vitro cytotoxicity against the same tumor.

Several models were discussed for adoptive chemoimmunotherapy in which transplantable, advanced, disseminated, antigeneic Friend, Moloney, and EL 4 leukemia in syngeneic or hemisyngeneic recipients were eradicated by a combination of CPP and syngeneic lymphocytes immune to the tumor. Greenberg found that the tumor

therapy was mediated by specifically immune H-2-restricted T-lymphocytes. Tumor eradication required a prolonged in vivo antitumor response susceptible to positive and negative influences. His depletion studies with monoclonal antibodies showed that successful therapy was mediated largely by donor cells which were noncytolytic Lyt $1^{+}2^{-}$ T-cells. The mechanism by which that T-cell subset mediates tumor destruction in vivo is not known but presumably requires the induction or amplification of another effector cell probably of host origin.

Fernandez-Cruz presented 2 models in which an advanced progressively growing antigenic syngeneic tumor was eradicated by infused immune cells and partly identified the critical effector T-cell. A large transplantable Moloney sarcoma and an MCA sarcoma growing in preirradiated BN rats were immunospecifically inhibited or totally eradicated by syngeneic lymphocytes primed to the target tumor in vivo and secondarily sensitized by 7-day cocultivation with tumor. The therapeutic results presented against tumors at least 1 cm in diameter were most impressive. The immune cells generated in vitro were separated in a fluorescence antibody cell sorter by 3 monoclonal antibodies which identify rat T-cells (W3/13) and their subsets, i.e., helper cells (W3/25+), and the cytotoxic/depressor cells (0X8). The W3/25+ cells, which were not significantly cytolytic to tumor in vitro, were therapeutically effective in vivo, whereas W3/25– cells were cytolytic in vitro but were not therapeutically effective in vivo. Indeed, they appeared to enhance tumor growth. The therapeutical effectiveness of W3/25+ cells coupled with the observation that after therapy the tumors continued to grow before ultimately regressing suggests that the critical donor effector cell acts as a helper cell in amplifying the response of the host and not simply as a directly cytolytic T-cell.

Purified IL-2 given to mice enhanced their cytolytic lymphocyte and NK responses in studies performed by Gillis. Cells from BALB/c mice immunized with EL 4 leukemia and then given IL-2 were significantly more cytotoxic in vitro than were controls when tested 5–10 days after immunization. The effector cells were T-cells, and their enhanced cytolytic lymphocyte activity was allospecific and did not represent polyclonal expansion of the cytolytic lymphocytes by IL-2; NK activity was similarly enhanced. Inasmuch as IL-2, produced by antigen or mitogen-activated T-cells, or both, is bound by other activated T-cells with resultant proliferation, T-cells primed by alloantigens in vivo should also bind IL-2, proliferate, and result in an increased cytotoxic response. Gillis' results support this view and represent the first direct evidence of an in vivo effect by exogeneously administered IL-2.

Cheever used models for adoptive chemoimmunotherapy of murine leukemias to study the efficacy of cells generated and maintained in vitro. Cells primed in vivo and secondarily sensitized with tumor in vitro were therapeutically effective. Such secondarily sensitized cells maintained in culture to 19 days with 12 days of IL-2 retained specific antitumor reactivity in vitro and were therapeutically effective in adoptive chemoimmunotherapy. The effect was immunospecific. Data presented suggested that the mechanism by which the long-term cultured cells eradicated tumor in vivo might be different from those of noncultured cells. On the possibility that the efficacy of such IL-2-dependent, long-term cultured cells might be limited by a lack of IL-2 in vivo, purified IL-2 obtained from Gillis was administered to tumor-bearing mice after chemotherapy and an infusion of cells which had been primed in vivo, secondarily sensitized in vitro, and maintained with it. The exogenous IL-2 clearly enhanced the efficacy of the long-term cultured immune lymphocytes.

When Paetkau cultured lymphocytes from DBA mice bearing P-815 mastocytoma for 5 days with tumor and IL-2, they became cytotoxic in vitro and had an antitumor effect when administered in vivo shortly after tumor inoculation. This effect did not appear to be immunospecific. Clones of T-cells, capable of killing P-815 in vitro, have been generated, but their in vivo efficacy has not been tested. The possibility that IL-2 has a direct antitumor effect in vivo was tested. Fraction 3 of IL-2 administered to tumor-bearing mice prolonged their survival. Confirmation of these results with purified IL-2 is important.

Rosenberg reported that murine lymphocytes sensitized in vitro to alloantigen and expanded and maintained in long-term culture in TCGF induced an immunospecific, second set-accelerated rejection of a skin graft. Similarly, spleen cells from C57BL/6 mice immunized with a syngeneic leukemia (FBL-3) in vivo, boosted with FBL-3 in vitro, and then maintained in TCGF for 2.5 months were significantly effective when used as an adjunct to CPP to treat C57BL/6 mice bearing advanced FBL-3. Therefore, sensitized murine cells (maintained in long-term culture and TCGF) exhibited in vitro activity in the transfer of adoptive immunity and in effective adoptive chemoimmunotherapy of leukemia. Rosenberg also observed that human lymphocytes grown in TCGF became cytotoxic to fresh autologous tumor but not to fresh autologous lymphocytes. However, the same cells killed virtually any cultured tumor or nontumor target. Thus by analogy to the report by Zarling, who made lymphocytes cytotoxic to autologous leukemia cells by alloimmunization to pooled donor cells, Rosenberg claimed similar generation of such reactivity for autologous nonleukemic solid tumors. The nature of the effector cell and the target antigens remains unknown. Although no data were presented, phase I trials with human lymphocytes are in progress.

North showed that progressive tumor growth in animals was associated with production or generation of suppressor cells which, in turn, caused a decay in concomitant antitumor immunity. This decay may explain the difficulty encountered when adoptively transferred immune cells are used in the eradication of an established tumor growing in a normal host. Immune cells were therapeutically ineffective in tumor-bearing mice which appeared immunologically normal, whereas immune cells were effective in mice preirradiated or T-cell-depleted by thymectomy, radiation, and reconstitution with bone marrow. Moreover, although immune cells alone had no effect and CPP alone had little on mice bearing advanced Meth A sarcoma, the combination of CPP and immune spleen cells was curative. In this and other models for adoptive chemoimmunotherapy, CPP may act to prevent the development of tumor-induced regulation of concomitant immunity.

The graft-versus-leukemia effect of alloimmunized H-2 compatible lymphocytes given to lethally irradiated AKR mice inoculated with AKR leukemia cells 1 day earlier was reported by Truitt. Cells from unprimed H-2 compatible donors had no graft-versus-leukemia effect, whereas cells from such donors immunized in vivo with lymphocytes from a variety of individual or pooled allogeneic mouse strains induced it. Results showed that such an effect could not be attributed exclusively to alloactivation by H-2 or HLA differences between donor and the alloimmunizing strain. Although the mechanism remains unknown, the results and those reports in which cells alloactivated in vitro have become toxic to tumor cells in vitro in man and animals suggest that cells alloimmunized in vivo and in vitro eventually have an antitumor effect in vivo.

Bach discussed the use of pooled alloantigens to generate cells cytotoxic to a syngeneic or autologous abnormal cell, e.g., a tumor cell. At the height of sensitization against targets such as RBL-5, mouse leukemia, or EBV-transformed autologous human lymphoblastoid cell lines, most of the antitumor cell immunity is mediated by cytolytic T-cells which are Thy 1^+ and Ly 2^+. Human pool-stimulated cells cytotoxic to autologous human lymphoblastoid cell lines were sensitive to OKT-3 or OKT-8 and complement. Results of studies with cytotoxic T-cells at the clonal level suggested that the basis of antisyngeneic or autologous abnormal cell toxicity following allosensitization is largely either sharing (as suggested by the model of alien histocompatibility antigens) or cross-reactivity between alloantigenic and tumor cell determinants recognized in the syngeneic combination.

The correlation between immunological function and cell surface phenotype of continuous murine cell lines was researched by Warner with the aid of a fluorescence antibody cell sorter. Thus normal splenocytes were cocultivated with irradiated BALB/c plasmacytoma (NPC-11) cells. After a secondary stimulation, the cytolytic lymphocytes were maintained for about a year with IL-2. Three anti-NPC-11 cytolytic lymphocyte lines were established: Types 2 and 3 were Thy 1.2^+, Lyt $1^-2^+3^+$. Also, 2 and 3 had an effect in the Winn tumor neutralization assay but had no effect when injected separately from the tumor. This failure was attributed to a defect in the homing mechanism, i.e., 70% of these lymphocytes were trapped in the lung and liver. However, normal animals may lack the appropriate activated cell subsets to provide the necessary cell interactions with cytolytic lymphocytes that result in an effective in vivo response. A continuous UV-induced suppressor T-cell line was also developed as were continuous NK cell lines. Cell surface phenotypes did not permit one to separate different T-cell populations from the normal lymphoid population. Moreover, Lyt 2 does not seem to be required either for antigen recognition or cytolysis.

Dennert cloned and subcloned an allospecific T-cell line and evaluated it for function and Lyt phenotype. Each of 3 sublines exhibited cytotoxicity, DTH, and helper activity with alloantigens, 3 functions originally assigned to 3 distinct subsets of T-cells. Furthermore, the correlation between function and Lyt phenotype was br ught into question because the cytotoxic cell line was Lyt 1^+2^-, whereas cytolytic T-cells are usually Lyt 2^+. Dennert suggested that the Lyt phenotype of T-cells may correlate with antigen recognition. Several discussants questioned the validity of the DTH reaction induced by the cell lines on the basis of kinetics, histology, and character, and also whether the helper activity displayed by the cell line was similar to that of other T-helper cells. Nevertheless, Dennert maintained that a given T-cell clone does not necessarily have a single function and that T-cells may appear to have a single function due to cyclic regulation of these functions through interactions with antigen or regulatory lymphocytes.

In his work on the antitumor reactivity in vitro and, to a lesser extent, in vivo, Kedar determined that cells from normal and tumor-bearing mice and from humans culture with TCGF. Over 20 lines of murine splenocytes cultured for 3–12 months were tested for reactivity against a large array of tumor targets. Without specific tumor stimulation, most lines were cytotoxic to most targets regardless of whether they were known to be sensitive or resistant to NK cells, lymphoid or nonlymphoid, fresh or cultured, autologous, syngeneic, or allogeneic. Even a given clone of effector cells reacted with a variety of tumor targets. Reactivity against syngeneic tumors and 1 allogeneic tumor in vivo was demonstrated only by the Winn tumor neutralization assay and by one for the elimination of radiolabeled tumor cells from the local site of inoculation of a tumor cell–lymphocyte mixture. Human continuous cell lines were also effective by the Winn assay against 2 tumor lines in athymic mice. The results showed that lymphocytes expanded and maintained with TCGF expressed a wide spectrum of cytotoxic reactivity. Cytotoxic cells appeared to be largely NK cells and possibly other natural effector cells or in vitro-activated lymphoid cells. The possibility was expressed that under appropriate conditions such cells are immunotherapeutically effective.

Ortaldo presented evidence that NK cells and typical T-cells from man can be cultured for the long term with IL-2. Human NK cells reportedly have a characteristic morphology of large granular lymphocytes. When these human lymphocytes were grown in the presence of IL-2 they possessed distinct cytotoxic capabilities. Cells highly enriched for immature large granular lymphocytes (as reflected by various criteria) were propagated in the presence of IL-2 and retained their morphology and cytotoxic reactivity. However, 7 to 10 days after initiation, these cultured lymphocytes became indistinguishable by surface phenotype from cultured T-cells in their reactivity to T-cell-specific OKT-3 antibodies. Unfortunately, the lack of stability of surface antigens during culture and the lack of NK-specific markers make the analysis of NK in culture most difficult. More selective cytotoxic cells might be generated and expanded with IL-2 if T-cells devoid of large granular lymphocytes are used instead of unfractionated peripheral blood mononuclear cells.

Several methods for deriving T-cell clones and how culture conditions will affect the functional properties of the clones were reviewed by Fitch. He emphasized that several different T-cells may be maintained simultaneously in long-term cultures stimulated with conditioned medium and that "only T-cells which have been cloned should be

considered to be cloned cells." Moreover, given the rapid rate of proliferation, variant cells are highly likely to emerge. The cloned cytolytic T-cells may have an extremely heterogeneous reactivity in regard to targets and may show lesser degrees of MHC restriction. The antigen recognition repertoire of noncytolytic T-cells may also be large, and a single noncytolytic T-cell clone may produce various biologically active factors, e.g., IL-2, CSF, MIF, IF, etc., and other lymphokines. Considerable heterogeneity has been noted in antigen reactivity patterns for cytolytic and noncytolytic cloned T-cells.

Nabholz presented results of studies on the use of cloned functionally active cell lines derived from cytolytic T-lymphocytes for a somatic cell genetics approach to the identification of the genes whose expression is required for the cytolytic lymphocyte-specific functions, such as target recognition, cytolysis, and IL-2 dependence. He derived a cytolytically active hybrid from a cross between inactive T-lymphoma lines and cytolytic lines selected in IL-2-containing media. Such hybrids can permit the identification of some of the chromosomes which carry genes that control the manifestation of the cytolytic lymphocyte phenotype. Nabholz's data suggested that several of the properties in which cytolytic lymphocyte and lymphoma lines differ from each other are controlled by one gene or several genes on a single chromosome.

A lymphokine termed "IL-3" was reviewed by Ihle. This IL-3, which is biochemically distinguishable from other lymphokines, such as IL-2 and CSF, is a product of activated helper T-cells, as are other lymphokines. Indeed, apparently all the Thy 1.2^+, Lyt 1^+2^- lymphocyte lines which Ihle developed constitutively produce IL-3, whereas T-cells of other phenotypes including cytotoxic lines do not. The IL-3 is not produced by B-cell mitogens or nonspecific activation of macrophages like IL-1. The unique and distinguishing characteristic of IL-3 is its ability to induce 20α-hydroxysteroid dehydrogenase, an enzyme with specificity for T-cell lineage, and nu/nu splenic lymphocytes in vitro. The induction is extremely rapid and does not require the generation of functional activity. Perhaps IL-3 affects an early step in T-cell differentiation that precedes the generation of functional activity and that, although the generation of cytotoxic lymphocytes requires IL-2 (because these lymphocytes are positive for the enzyme), their optimal generation may require both types of IL. Data were presented that several phenotypes of lymphocytes required IL-3 for growth in vitro. The requirements for IL-3 for growth ultimately helped define a specific lineage of lymphocytes.

Greene investigated the suppressor cell network of dealing in the response, not to tumor antigen, but to the hapten azobenzenearsonate. In his models, DTH or cell-mediated cytotoxicity to the azobenzenearsonate cell surface structures can be readily induced as can antigen-specific suppressor cells. The studies presented emphasize the existence not of a single suppressor cell but a heterogeneous suppressor cell population which contains cells from various Lyt subsets and involves a series of steps, each of which may potentially be amenable to modification. Some suppressor cell populations and factors can suppress the generation of cell-mediated cytotoxicity by cytotoxic T-cells, not by interacting with these cells or their precursors but probably by inhibiting or influencing the delivery of signals from helper T-cells necessary for the induction of the cytotoxic ones. Nonidiotypic immune responses were also discussed. The signaling and recognition processes which take place among suppressor cells may be independent of the importance of idiotype regulation in the response to that antigen. That action implies the possibility of intervention into the suppressor network which can be applied to any antigen. Thus a knowledge of the molecular structure of the products of specific subsets of suppressor T-cells and how the molecules function, the identification of the products of the effector suppressor cell, and an understanding of the role of I–J in suppressor signals are likely to provide direction for potential manipulation of the suppressor network for possible antitumor immunotherapy.

A review of the identification, differentiation, and function of human T-cell subsets was provided by Reinherz. Selective T-cell subset manipulation for potential immunotherapy requires a dissection of the cellular mechanisms and interactions in the generation of human T-cell responses. This has been greatly facilitated by the development of in vitro methods for our characterizing, identifying and purifying human T-cell subsets by cell surface markers and for discriminating functional properties and interactions of the isolated T-cell subsets with other cells. Excellent correlation was reported between particular function and possession of certain glycoprotein antigens identifiable by the monoclonal antibodies. For example, inducer T-lymphocytes have the T-4 antigen, but suppressor and cytotoxic T-cells bear T-5. Indeed, there is an association among cell surface phenotype and function and T-cell subsets in man and those in the mouse. For example, the $T\text{-}4^+$ subset in man is analogous to the Lyt 1^+2^- subset in the mouse that provides helper function to specific and nonspecific signals and also induces suppressor cell activation, whereas the $T\text{-}5^+$ subset in man is analogous to the murine Ly 2,3 subset, which mediates cytotoxic and suppressor functions.

Fatham discussed findings showing that clones of murine T-cells can be obtained that can recognize soluble antigen in the context of I-region-restricted recognition. The antigen-specific proliferation could be maintained in vitro only by antigen stimulation in the context of I–A-compatible presenting cells. His data documented the existence of unique hybrid Ia molecules present on cells from F_1 heterozygous mice. The hybrid products were considered to be functional counterparts of the conventional I–A product recognized on the cells from homozygous mice. He suggested that T-cells be selected, perhaps in the thymus, for recognition of the expression of I-region products and that it may be important to determine whether these hybrid molecules exist in man as well as in the mouse, in view of the implications of attempts to reconstitute man with T-cell clones.

APPENDIX

INTRODUCTION

The recently coined term "biological response modifiers" includes the many agents and approaches that have a mechanism of action which involves the individual's biological responses. Although activation of the host response, particularly the host immune response, has been the goal of therapists for many generations, recent technological advances have improved our understanding of biological responses and our ability to manipulate them. In particular, tremendous strides have been made in the last 3–5 years in molecular biology with respect to nucleic acid sequencing and translation, protein sequencing and synthesis, isolation and purification of biological products, mass cell culture, and genetic engineering of eukaryotic genes into bacteria and yeast. The advent of these new technologies has led the way to a greater understanding of biological compounds which may exist in exceedingly minute quantities but have tremendous amplifying powers with respect to their effect on host responses. Historically, activity has been detected in many biological preparations but the isolation and purification of the active factor has been fraught with many difficulties. The technology is now here for large-scale production of biological products, the isolation and purification of these products, and eventually their production by genetic engineering or by chemical synthesis. Because of these recent advances, biological response modifying therapy is now a reality, and our understanding of BRM will likely be of great value in our comprehension of cancer biology and of cancer therapeutics in this decade.

The BRM Program is a comprehensive program of the Division of Cancer Treatment, NCI, involved in clinical and laboratory research with both extramural and intramural components to investigate, develop, and bring to clinical trials potential therapeutic agents which may alter biological responses important in the biology of cancer growth and metastases (*1*). This Program was conceived as a focused approach in the Division in support of further basic research in BRM and for rapid application of potential leads from that research to the treatment of cancer in man. The classes of agents to be investigated in this Program include immunoaugmenting, immunomodulating and immunorestorative agents, IF and IF inducers, lymphokines, cytokines, and growth factors, thymic factors, tumor antigens, and modifiers of tumor antigen cell surface components, antitumor antibodies, antitumor cells, tumor-preventing agents, and maturation and differentiation factors. Considerable research is underway in each of these areas but a focused, coordinated approach by the NCI may result in the rapid acquisition of knowledge and a more rapid application of information to the treatment of cancer.

BACKGROUND

Personnel at the NCI have been evaluating the potential role of biological response modifying agents in the treatment of cancer over the past several years. In March 1975, Dr. Frank Rauscher, then Director of the NCI, established the Interferon Working Group to "monitor developments in interferon." The following November, the National Cancer Advisory Board recommended that the NCI purchase IF for basic clinical studies. That same month, a report to the Division of Cancer Treatment, Board of Scientific Counselors,[1] by Dr. Mathilde Krim recommended that IF be further investigated as an antitumor agent. The Board recognized the emerging importance of biological response modifying agents and directed the staff of the Division of Cancer Treatment, NCI, to monitor this important new field carefully.

In mid-1976, the NCI purchased human leukocyte, lymphoblastoid, and fibroblast IF through the Division of Cancer Biology and Diagnosis. Six investigators were given IF for clinical trials, and 33 investigators received them for basic laboratory research. The Board of Scientific Counselors was again presented with information on the clinical use of IF as an anticancer agent in October 1976, with a review of the Karolinska Institute trials.

The increase in availability of IF and the recognition that it might be a useful antitumor agent prompted the NCI to ask the Board of Scientific Counselors of the Division to review the data on IF as well as other biologicals that might have some influence on tumor growth and metastasis. In October of 1978, the Board recommended that a more concerted BRM Program be developed within the Division of Cancer Treatment. The BRM Program began with the appointment of the Subcommittee on Biological Response Modifiers (*see* p. 4) by the Board of Scientific Counselors in October 1978. This Subcommittee was established because of the recognition that BRM were destined to play an increasing role in the treatment and understanding of cancer. The initial charge of this Subcommittee was to review existing clinical and laboratory data and ongoing investigations to develop guidelines for a focused program within the Division that would subsequently encourage, support, and direct the NCI effort in this area.

This Subcommittee completed the difficult task of reviewing the BRM background information and made programmatic recommendations in their interim report of September 1979.

Through a series of meetings among the Subcommittee members, workshops organized by the Subcommittee, informal discussions with scientists conducting research in the area, extensive travel to and attendance at scientific meetings on BRM, and through extensive contacts with consultants with expertise in the laboratory and clinic relevant to these agents, the Subcommittee completed an interim report for the Board of Scientific Counselors. This interim report defined BRM as those agents or approaches which modify the relationship between tumor and host by modifying the host's biological response to tumor cells with resultant therapeutic effects. Included in this definition were several approaches: 1) increase the host's antitumor responses through augmentation and/or restoration of effector mechanisms or decrease that component of the host reaction which may be deleterious; 2) increase host defenses by the administration of natural or synthetic

[1] Reference to "the Division" should be interpreted as the Division of Cancer Treatment. This Board of Scientific Counselors serves this Division only.

effectors or mediators; 3) augment host responses to modified tumor cells which might stimulate a greater host response or increase tumor cell sensitivity to an existing response; 4) decrease the transformation or increase a differentiation (maturation) of tumor cells, or both; and 5) increase the ability of the host to tolerate damage to cytotoxic modalities of cancer treatment.

The interim report of the Subcommittee consisted of two documents. The Appendix to the interim report reviewed the pertinent literature and provided scientific background on BRM. In this selective review, the Subcommittee attempted to identify promising approaches from previous studies with these agents. The interim report described the Subcommittee's view of how the BRM Program should be organized, initiated, and administered. The final version of the Subcommittee's report is the substance of this Monograph.

As the Subcommittee deliberated, investigators in the Division of Cancer Treatment began to identify resources to support laboratory research and clinical trials of BRM. An interim administrative program was established in the Office of the Director of the Division, and a search for the Director was initiated. The Subcommittee was consulted frequently by Program Staff for assistance in decision-making relative to the initial organization of the BRM Program and to the design and initial organization of BRM research contracts, grants, and clinical trials. As a result of these deliberations, the Division and the NCI made available 13.5 million dollars in fiscal year 1980 to establish the BRM Program and to begin initial work. Initially, emphasis was primarily on IF and IF-related research. Lesser, though substantial, resources were applied to other BRM.

The Director, BRM Program, began working at the NCI in October 1980, and the semifinal report by the BRM Subcommittee was presented to the Board in the same month. In consultation with the Division of Cancer Treatment and those Program officials involved in the initial organization, the Director set up and organized the Program according to the guidelines established by the BRM Subcommittee. This report describes the progress to date and the future initiatives currently under consideration at the NCI.

PROGRAM OBJECTIVES

The objectives of the Program are:

Establishment of a well-focused program within the Division of Cancer Treatment to promote laboratory and clinical research in BRM.

Support of both basic and clinical research in the extramural community through a balanced program of grants and contracts: Program Announcements, RFA, and RFP will be used to support these efforts.

Establishment of the BRM Program as a resource for both the extramural and intramural communities to provide certain BRM for laboratory and clinical use in further investigations of mechanism of action and therapeutic efficacy.

Establishment of a screening program and a BRM Decision Network similar to that now in effect for drug development in the Division. Both a broad-based screening program to identify potentially active agents (common track) and assays of more specific BRM (specific tracks) will be developed to assist the Program Decision Network Committee in making judgments about which BRM should be pursued through further preclinical work and formulated for clinical trials.

Establishment of an intramural laboratory and clinical program for the pursuit of investigations on basic mechanisms of action of BRM in humans, animal tumor models, and in vitro assays.

Production of BRM through the genetic engineering and fermentation resources of the Program for potential use in clinical trials in man.

ORGANIZATIONAL STRUCTURE AND FUNCTIONAL DESCRIPTION OF THE BIOLOGICAL RESPONSE MODIFIER PROGRAM

Organizational Chart

The BRM Program was initially set up in the Office of Director of the Division of Cancer Treatment. Subsequently, Dr. Vincent T. DeVita, Director, NCI, requested that it be a Program within the Division of Cancer Treatment. The Program was officially approved as a Division program by the then Secretary of Health and Human Services Richard Schweiker in April 1981. The organizational structure of the Division of Cancer Treatment is given in figure A-1, and the organizational chart for the BRM Program is shown in figure A-2. The extramural branch has been designated the Biological Resources Branch, and the intramural branch is the Biological Therapeutics Branch.

Program Director

The Director, BRM Program, has been designated as an Associate Director, Division of Cancer Treatment, and has been given the responsibilities equal to that of the other four Associate Directors. The Director has responsibility for planning, directing, and coordinating all preclinical developmental research and clinical trials and related studies pertaining to this Program. He is responsible for planning and directing a total research effort, extramural and intramural, directed toward the assessment of the efficacy of BRM and their use in the treatment of cancer. The Director serves as the focal point for collaborative efforts between the Division of Cancer Treatment and other NCI components, other Institutes of the National Institutes of Health, and public and government agencies for this Program. He also serves as the senior NCI staff advisor to the Director, NCI, and the National Cancer Advisory Board in the area of BRM. He will develop and formulate policies and plans for the BRM Program and establish cooperation among various governmental and nongovernmental organizations. As spokesman for NCI with the scientific community, the Director, BRM Program, will organize specialized workshops to introduce the Program to the scientific community and to gain support for it. The Director serves under the general supervision

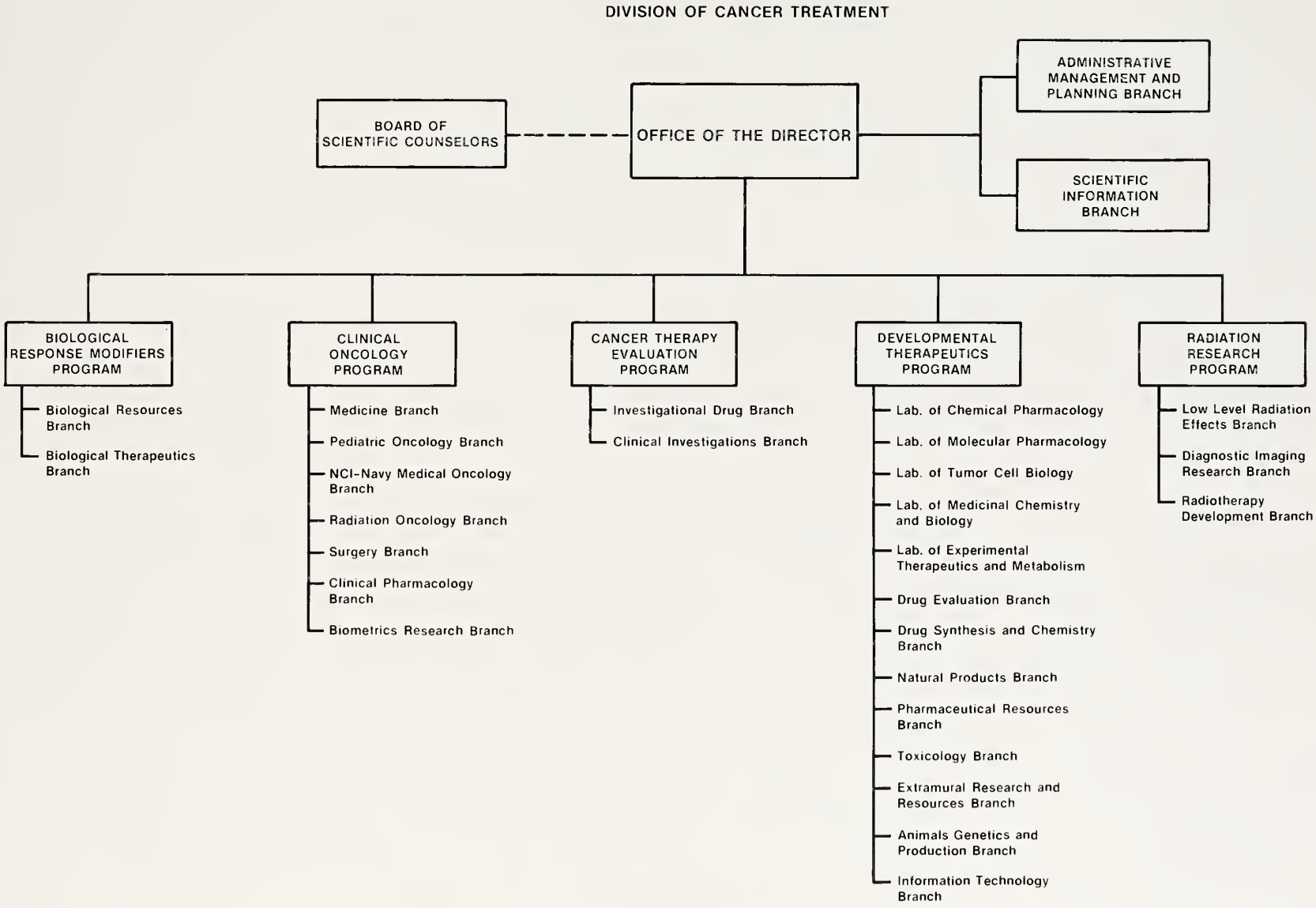

FIGURE A-1.—Organizational structure of the Division of Cancer Treatment.

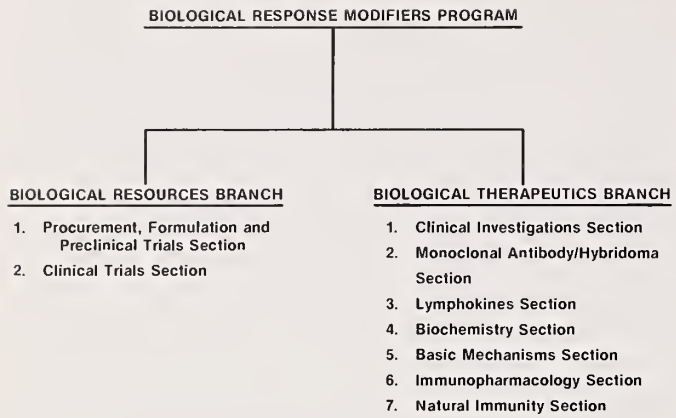

FIGURE A-2.—Organizational chart for the BRM Program.

and guidance of the Director, Division of Cancer Treatment. He is expected to take initiative and will execute his responsibilities with minimal supervision. Work is evaluated according to fulfillment of the broad program objectives and national goals, the effect of his advice and influence in managing and achieving a quality research and clinical program, and contributions to the advancement of research and development of new effective treatments of cancer with BRM.

Program Description

Extramural Program: Biological Resources Branch

This Branch is responsible for all extramural activities of the BRM Program including BRM development, preclinical trials, and evaluation and clinical trials. It is also responsible for the administration of all BRM Program grants and contracts. It is comprised of 2 sections: the BRM Procurement, Formulation, and Preclinical Trials Section and the BRM Clinical Trials Section. The Preclinical Section is responsible for the identification of BRM of interest to the Program, review of the literature with respect to potential activity of such agents, and dissemination of such information to others in the Program, so that these agents can be considered for further evaluation. Compounds may come from individuals, research groups at universities, and the pharmaceutical industry. This Section has the responsibility of relaying this information to the BRM Program Operating and Decision Network Committees. The Operating Committee acts as a steering committee for the Network Committee and reviews each potential compound or approach as to feasibility, practicality, and scientific interest. After initial consideration by the former group, the recommendation can be made to present the agent or approach to the Network Committee for a decision as to further evaluation.

This latter Committee is larger and formally considers which agents should receive further evaluation through the Program. It has a broad-based constitution, with representatives from many areas of expertise related to the BRM Program, including those from the various specialities at the NCI, as well as other Institutes in the National Institutes of Health and consultants from outside. Selected agents or approaches are presented to the Network Committee for appropriate discussion by the primary and secondary reviewers. After a consensus is reached, the agent or approach may be evaluated in further trials. A recommendation is made as to whether additional background work is needed or whether the agent or approach can be directly evaluated in the BRM Program screen or by other specific assays in the Program, or both. For example, should an immunomodulator be formulated by a pharmaceutical company, this compound may be presented to the Program with background information on its activity. The Network Committee would determine whether sufficient background data are available to make a determination on the compound. If an affirmative decision is reached, the compound would then be recommended for further investigation in the Program (fig. A-3).

A major charge for the scientists in the Biological Resources Branch is to develop a screening program to detect active biologicals in the treatment of cancer. A screening program has been formulated based on the drug screening program and on the recommendations of the BRM Subcommittee, as well as those of many experts in the field. This screening program is discussed in detail below.

The Procurement, Formulation, and Preclinical Trials Section of this Branch is responsible for following each of these agents through preclinical trials, the Committee decisionmaking process, the screening programs, the procurement and formulation phases, and finally up to the clinical trials phase for each particular agent or approach. Those compounds of potential interest (active in the BRM screening program) will be tested in the regular drug screen program with transplantable tumor systems normally used for drugs. Thus most response modifiers will have been tested in the BRM screening program and, if active, will also have been tested in the drug development screen. Correlations can then be made with activity in each screening program and subsequent clinical activity. Toxi-

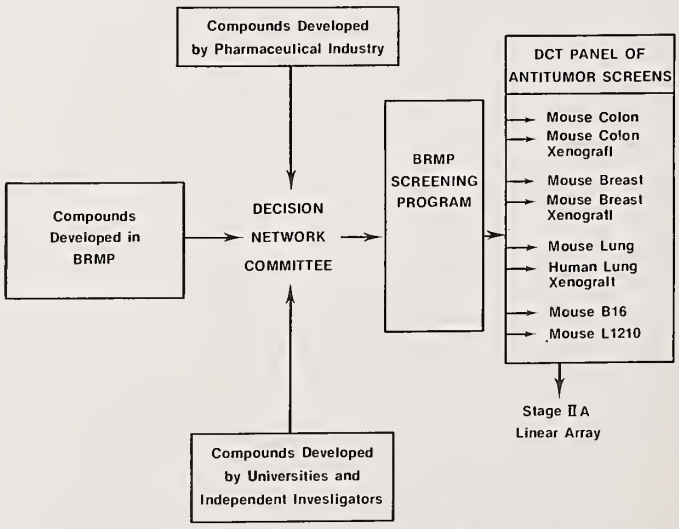

FIGURE A-3.—Development of BRM. DCT = Division of Cancer Treatment; BRMP = BRM Program.

cology, when appropriate, will be done with the same systems used for drug toxicology evaluation. New agents entering clinical trials will have had this broad background of evaluation with respect to activity and toxicity. Many agents proposed for testing by the BRM Program may lack sufficient background data to go through the evaluation described above. The Network Committee and Program staff may recommend to the initiating investigator or institutional group that further testing be done before resubmission to the BRM Program.

The Clinical Trials Section will monitor Program clinical trials and administer clinical grants and contracts. Each agent or approach which has gone through the screening and evaluation procedures described above will then be recommended for clinical trials by the BRM Program to the Decision Network Committee; a determination as to testing will be made with respect to mechanism for these clinical trials. Most potential agents will be tested under the "task order mechanism" through the extramural clinical trials program. These kinds of trials have already been completed for IF, thymosins, and MVE-2. The mechanisms through which investigators obtain approval from the Office of Biologics, Food and Drug Administration, advertise and initiate a clinical trial, design and monitor such clinical trials, and the mechanisms for data acquisition and interpretation are already functional in the CTEP. The Clinical Trials Section for the BRM Program will be involved in the monitoring of the clinical trials under the Program but will work with the existing mechanisms and personnel in the Biological Evaluation Branch, CTEP. Monitoring of clinical trials is anticipated with personnel from CTEP and the BRM Program working jointly to conduct and manage the Division of Cancer Treatment extramural clinical trials with BRM. Such an arrangement is already in place for our current task orders.

Intramural Program: Biological Therapeutics Branch

The Office of the Director, BRM Program, the intramural laboratory, and clinical facilities are all located in or near the NCI–FCRF in Frederick, Maryland. Several areas of interest are to be pursued in the intramural program. Most involve basic investigations into potential new approaches to the treatment of cancer. The clinical activities will be administered in a small facility at Frederick Memorial Hospital. This 245-bed hospital is located just a few blocks from the FCRF. Initially, a 4-bed inpatient clinical unit for the evaluation of BRM was developed at this hospital. We anticipate this will expand to 10 to 15 beds in the future. A small 10-bed outpatient clinical facility has also been established to allow for outpatient treatment in the Medical Pavilion adjacent to the hospital. Support facilities include a leukopheresis and plasmapheresis unit, expanded services from the staff of the Pathology Department to provide expertise and assistance in the monitoring of patients undergoing trials with BRM, and expanded radiological services including a head and body computed tomography scanner for the evaluation of these patients. The clinical facilities opened in April 1981, and provide a designated Clinical Oncology Research Unit for evaluation of new BRM in phase I–II trials. In addition to the intramural clinical activities performed at Frederick Memorial Hospital, cooperative programs for the evaluation of BRM have been developed throughout the NCI intramural clinical system including the Clinical Center and the Bethesda Naval Hospital. The working group to develop cooperative intramural protocols has been established. The initial clinical trial is underway and utilizes genetically engineered recombinant leukocyte A IF (Ro 22-8181), supplied by Hoffmann-LaRoche, Inc. (Nutley, N.J.). This phase I toxicity trial began in April 1981 and was completed in January 1982 (*2*). Investigators have recently begun the phase II trials to determine the anticancer activity of this agent.

BIOLOGICAL RESPONSE MODIFIER SCREEN: DETECTION OF BIOLOGICAL ACTIVITY IN PRECLINICAL MODELS

Since the late 1800's, numerous researchers have focused their efforts on eradicating neoplastic diseases by the manipulation of the host with agents now classified as BRM. An implicit assumption in these studies has been the belief that clinical cancer is the consequence of altered hemostasis by which host responses to an oncogenic challenge are diminished or absent. Thus it was reasoned that the successful awakening or boosting of a host's response to neoplasia could lead to tumor regression. However, clinical immunotherapeutic trials with a variety of agents yielded discouraging results that were greatly inferior to those obtained in various animal models.

These poor results may have several causes. In general, animal tumor systems relied on the use of neoplasms transplanted into *normal* syngeneic recipients. In addition, many of those conducting these studies were actually investigating prophylaxis because they involved stimulation of the host before or simultaneously with tumor implantation. Few data are available on the ability of syngeneic animals to reject established metastases, and even fewer are available on the outcome of immunotherapeutic studies of metastasis in animals bearing autochthonous malignant neoplasms.

Central to the identification of BRM agents useful for clinical oncology is the recognition that, in the main, the challenge in humans is the eradication of metastases that occur in the primary host. In this regard, two important facts must be kept in mind. First, metastases can result from the proliferation of many different subpopulations of cells that reside within the primary neoplasms (*3, 4*). This may explain the findings that cells residing within an area of metastasis can be antigenically distinct from their parental tumor (*5–14*) or from various other metastases (*11, 12, 15, 16*). The implications of such findings as they relate to the outcome of specific immunotherapy are obvious (*7, 17, 18*). Second, normal animals are not and should not be assumed to be comparable to animals bearing autochthonous neoplasms (*7, 19*). Specific or nonspecific defects may exist in humans and animals that lead to the development of their autochtonous tumors. Corrections of such defects may require a totally different form of biological modification than that required to assist

the normal host in controlling neoplastic growth and metastasis.

Criteria for Successful Screening

The screening of a large number of BRM for their therapeutic potential is, upon first consideration, an awesome task. Built into the design of such a testing program should be the ability for us to survey a wide range of defined biologicals and relatively pure chemical materials in a highly controlled environment. Ideally, the in vitro assays used should be predictive of the ability of a given BRM to modulate in vivo the host's innate biological responses to cancer beneficially. The recommendation that an agent be tested clinically should be based on evidence obtained from meaningful in vitro or in vivo studies, or both, that demonstrate that a BRM will aid the host in either the prevention or eradication of metastasis.

Theoretically, an ideal procedure for screening BRM for their therapeutic potential should involve a system of sequential and progressively more demanding studies designed so that a maximum number of effective agents can be selected. Those agents with demonstrated broad-spectrum activity, which perform well in the preliminary first-line assays, would be tested in a more specific and discriminating manner. Once proven successful in a second-line assay, the agents should be tested in a third and more complex assay system and so on.

The term "common track" denotes a series of sequential assays through which many BRM agents are screened for therapeutic potential. By no means do we suggest that all potentially effective BRM must successfully pass the common track screen. For some BRM such a screening procedure may be inappropriate. For the testing of many of these BRM, the development of suitable specific tracks will be required (fig. A-4). For example, one would not determine the activity of a monoclonal antibody with antitumor specificity by using the common track screen. Rather, a specific system that required antibodies, tumor cells, and non-cross-reactive control target cells would be used. The common track schematic for this type of selective and orderly preclinical screening of newly defined chemical or biological augmenting agents is shown in figure A-5. Such a step-by-step approach to the screening of potential BRM has been designed so we could define their effects on T-, B-, and NK-cell, and macrophage function. The proposed sequence of progressive assays is: 1) in vitro activation:in vitro testing; 2) in vivo activation:in vitro testing; and 3) in vivo activation:in vivo testing. This sequence allows exploration of the variables of dose, schedule, route, duration and maintenance of activity, adjuvanticity, and synergistic potential in an orderly fashion for each BRM.

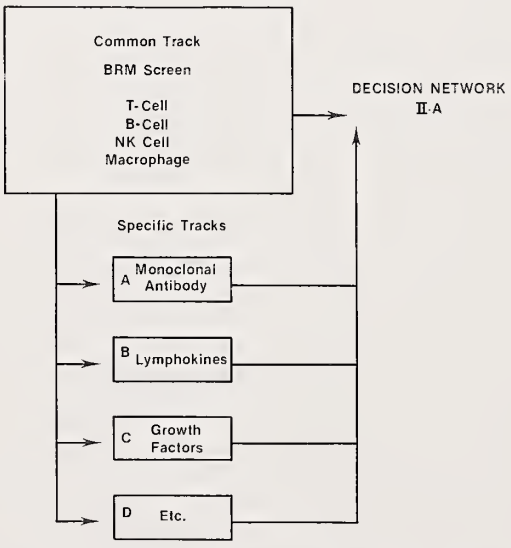

FIGURE A-4.—Screening program of the BRM Program.

Initially, one should use normal donors in assays to define the base-line criteria for a positive response and to exclude the influence of tumor-induced or tumor-associated suppressor cells. The next series of assays would be done on effector cell populations isolated from animals bearing transplantable tumors with metastatic disease. Finally, because normal animals bearing transplantable tumors are not comparable to animals bearing primary tumors, another series of assays would be performed in autochthonous tumor systems which may provide the most relevant models for studies of BRM. Agents found promising in their ability to increase resistance to a tumor challenge would be screened further for their ability to prevent or eradicate experimental metastasis in normal and immunosuppressed hosts. Furthermore, advantage will be taken of the recent finding that 3-week-old nude mice, whose levels of NK cell activity are low, develop metastases from injections of allogeneic and xenogeneic tumors such as human melanoma and colon carcinoma (*20–22*). This likelihood of the young nude mice to develop metastases can be readily reversed by a pretreatment with BRM that activate NK cells. Thus investigators can use this model to assay for agents that activate or augment NK cells. Finally, at this level of the screening program, agents will be evaluated for their ability to eradicate spontaneous metastases produced by tumors growing in a primary site after they were implanted into syngeneic animals. Ultimately, agents found effective in these hosts will be evaluated in rodents bearing autochthonous neoplasms.

Efficacy Testing of Promising Biological Response Modifiers in Primary Hosts

The preclinical evaluation of BRM requires the testing of these agents in relevant in vivo model systems. We cannot overemphasize the importance of using primary hosts for investigating BRM that show preliminary therapeutic potential in transplantable animal tumor models. Although this concept is generally recognized and accepted by researchers, the ability to obtain significant numbers of primary hosts in a reasonable period after chemical or physical carcinogenesis remains a problem. Truly spontaneous neoplasms (of unknown cause) arise in rodents, but the use of these tumors as models is currently not feasible.

The UV radiation carcinogenesis model developed by Kripke and co-workers (*23–26*) may prove to be useful for

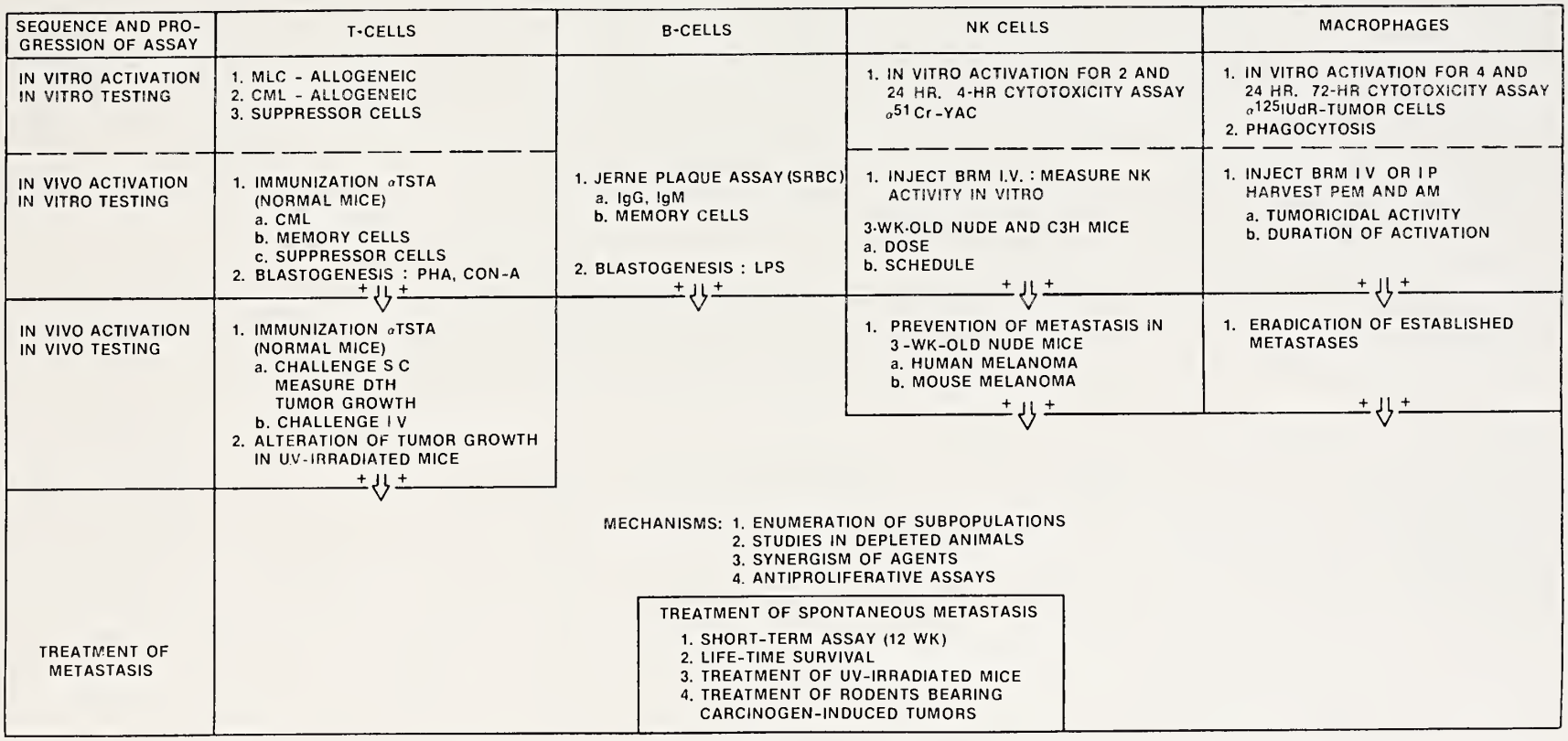

FIGURE A-5.—Flow chart for preclinical screening of potential BRM. IUdR = iododeoxyuridine; TSTA = tumor-specific transplantation antigen; PEM = peritoneal exudate macrophages; AM = alveolar macrophages.

studies of the effectiveness of BRM in primary hosts. In this system, chronic exposure of mice to UV radiation results in the development of single or multiple skin neoplasms. These tumors are antigenic, and most are rejected upon transplantation to normal syngeneic recipients. However, the tumors grow progressively in immunologically deficient recipients. In addition, UV-induced neoplasms grow progressively in syngeneic mice that have been exposed to low-dose nontumorigenic UV radiation. Studies of this phenomenon revealed that the inability of UV-irradiated mice to reject challenges with syngeneic UV-induced tumors is due, at least in part, to the presence of suppressor T-cells in their lymphoid organs (*27, 28*). The reactivity of the UV-induced suppressor cells is antigen restricted; these T-cells do not suppress the rejection of either allogeneic UV-induced tumors or even syngeneic chemically induced tumors (*25, 26*). Furthermore, the immune response of UV-irradiated mice to various exogenous antigens is normal (*26*), which suggests that the suppressor cells show selectivity for antigens expressed on autochthonous UV-induced tumors.

Obviously, if we are to assess the full spectrum of a BRM accurately and systematically, we need to test the antitumor activity of such an agent in more than one system of primary hosts. Therefore, it is essential that efforts be accelerated toward development of other chemical carcinogen-induced models. The ideal carcinogen-induced tumor system would be one in which the carcinogen is easily administered, has a short latent period, is not highly toxic, and is capable of reproducibly inducing palpable primary tumors that metastasize in a high percentage of rodents. The induction of mammary tumors in rats by *N*-methyl-*N*-nitrosourea appears to be a suitable carcinogen-induced tumor system with many of these characteristics (*29, 30*). We are currently investigating this system for BRM screening.

Expectations and Goals

A viable screening system for BRM must be adaptable to changes as data begin to accumulate. It is important for all to recognize that screening programs for chemotherapeutic agents were initiated by the NCI in the mid-1950's (*31*). The initial program and its evolution are shown in table A-1, and the current drug screening program is depicted in figure A-6. Clearly, the drug screening program has been sensitive to developments in the field of tumor cell biology, as is illustrated by the recent addition of the human tumor stem cell assay as part of the screening procedure. Initially, those in the drug screening program attempted to examine literally thousands of random compounds for antitumor activity (*32*). It is unlikely that such a large screening program for BRM could or should be initiated, given our present resources and inclinations. Rather, this program is designed to screen and test agents for which some background information concerning proposed mechanisms of action or pilot data, or both, regarding efficacy is available. The BRM screen should confirm, standardize, and extend observations in a valid, systematic, and interpretable way to provide a vehicle for the translation of data to the clinical reality. Those BRM that are promising will be tested for possible toxicity. Therefore, our process of evaluation for BRM is similar to that already in place

TABLE A-1.—*Evaluation of NCI in vivo screens*

Year	Tumor models
1955	Sarcoma 180, carcinoma 755, leukemia L1210
1960	L1210 and two models from a pool of 21
1965	L1210 and Walker carcinosarcoma 256
1968	L1210 for synthetics L1210 and leukemia P388 for natural products
1972	L1210 for synthetics; P388 for natural products B16 melanoma and Lewis lung carcinoma for special testing
1975	Broad-spectrum screening against mouse tumors and human tumor xenografts of major tumor types as well as models of known value

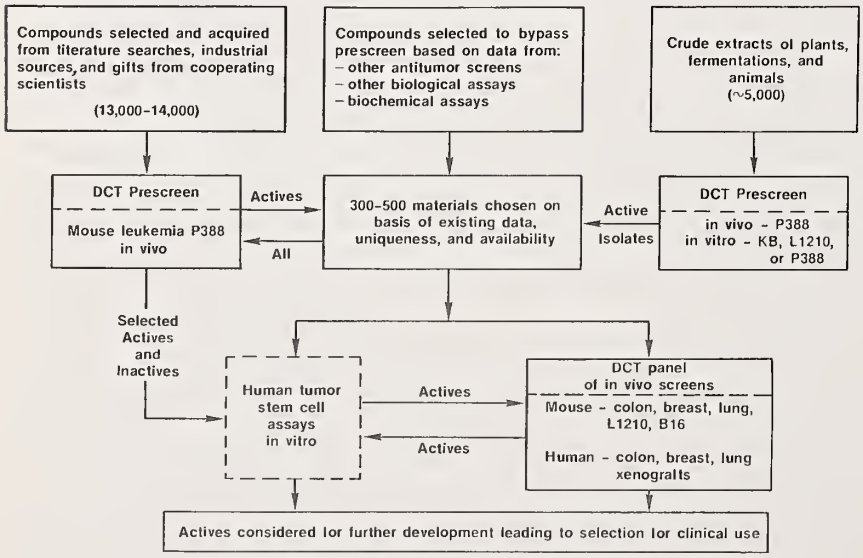

FIGURE A-6.—Flow of drugs through the Division of Cancer Treatment screens, 1980.

for drugs. Moreover, we also plan to compare our screening program to the drug screening program by testing agents active in the BRM screen in transplantable tumor systems currently used for screening of chemotherapeutic agents.

We are aware of the difficulties and the temptations to extrapolate data obtained from in vitro assays to the in vivo models and on to the clinical reality of cancer therapy. Clearly, many BRM may be highly successful in activating host responses in vitro but have no similar function in vivo. Alternatively, those BRM agents that are dependent on the host's metabolism to become active or require the interaction of several host components are likely to yield positive results when tested in an in vivo model but may be negative in the preliminary in vitro assays. For these reasons, we plan initially to test many BRM in all the in vitro and in vivo assays and then examine their therapeutic efficacy.

Initially, we anticipate that only 20 to 40 BRM per year can be tested in our experimental screening program. Essentially, we shall first test the screening procedure by examining the activities of several known BRM. This will allow us to determine in a systematic way which tests are predictive and which are not for the therapeutic benefits of a BRM. By concentrating our efforts on data-based and proved assays, one should be able to increase the number of BRM screened for possible clinical use to meet the criteria shown in tables A-2 and 3.

Successful approaches to immunotherapy for disseminated cancer should be more forthcoming once proper consideration is given to the pathobiology of metastasis. The outcome of the process depends on both host and tumor properties, and the balance of these relative contributions may vary among tumor systems (*3, 7*). This variation suggests that useful tumor models, designed to answer clearly defined questions, can be selected by consideration of this interplay of tumor and host properties.

Whether induced or transplantable animal tumor systems, of which there are many, are valid models for therapeutic modalities for human cancer has been a controversial issue (*33–37*). It is important for one to remember that, even in humans, therapy successful for one type of tumor may not be successful for another type. The failure of animal tumor models to serve as predictive models for human cancer does not necessarily diminish their usefulness but rather reflects our choice of an unsuitable model to answer a specific question (*19*). Another major problem is that, unlike the model systems in which treatment can be given with precise timing relative to the metastatic phase of an implanted tumor or injected tumor cells, clinical diagnosis of cancer is generally late, and micrometastases and often macrometastases are established and growing before treatment can be initiated.

TABLE A-2.—*Attributes of a successful screen*

Validity
Specificity
Predictability
Clinical relevance
Cost and time efficiency

TABLE A-3.—*Clinical relevance of a screening program*

Classification	Screen	Clinical trial
True positive	+	+
False positive	+	−
True negative	−	−
False negative	−	+

Most important is the establishment of a strong scientific base upon which the principles of biological response modification can be built and subsequently expanded. The sequence of investigations outlined here is aimed directly at the formation of such a data base at the preclinical level. We believe that the lessons learned from previous screening attempts have been useful in formulating this approach, which represents a synthesis of many views and recommendations. It is anticipated that the testing of BRM in an evolving, controlled system will help eliminate arbitrary decisions with regard to preparation and use of a given BRM and ultimately will contribute to the development of novel approaches for the treatment of disseminated cancer (*38*).

CLINICAL TRIALS

Clinical trials with BRM require a different approach than that traditionally used in tests on new drugs. Whereas drugs are initially tested for toxicity and then for antitumor activity, BRM must also be tested for their ability to modify the appropriate biological response (*39*). Still there are many similarities in our overall approach to that used in drug development. We adapted the linear array approach to conceptualize our approach (fig. A-7). We need to establish the MTD and the maximum biological response modifying dose (*39–41*). These concepts require verification in clinical trials and such trials are currently underway. The BRM Program primarily funded extramural research for fiscal year 1980 from its allotment of 13.5 million dollars. A considerable portion was used in the acquisition of various IF and in testing them and other BRM in clinical trials. Each of the projects initiated in 1980–81 is listed in table A-4. A major new initiative was taken in establishment of the master contract–task order mechanism for use under the BRM Program. Although this mechanism has not been extensively tested by the NCI for the conduct of clinical trials, we considered it the most flexible mechanism available to institute quickly and efficiently the clinical trials in this rapidly evolving area. In 1980, tasks were funded in 12 universities (table A-5) of the 27 approved under the initial master contract competition (table A-6).

Most (76%) of the 1980 funds were expended in the extramural area with most of these resources directed at the IF-related research, procurement, and clinical trials. Considerable emphasis continues on IF, but a broader approach is being developed with support for the thymosins, retinoids, lymphokines, antisuppressor cell monoclonal antibody, and antitumor cell monoclonal antibody, in fiscal year 1981. New task order contract initiatives for 1981–82 include: monoclonal anti-T-cell antibody in T-cell cancers and antisuppressor cell monoclonal antibody

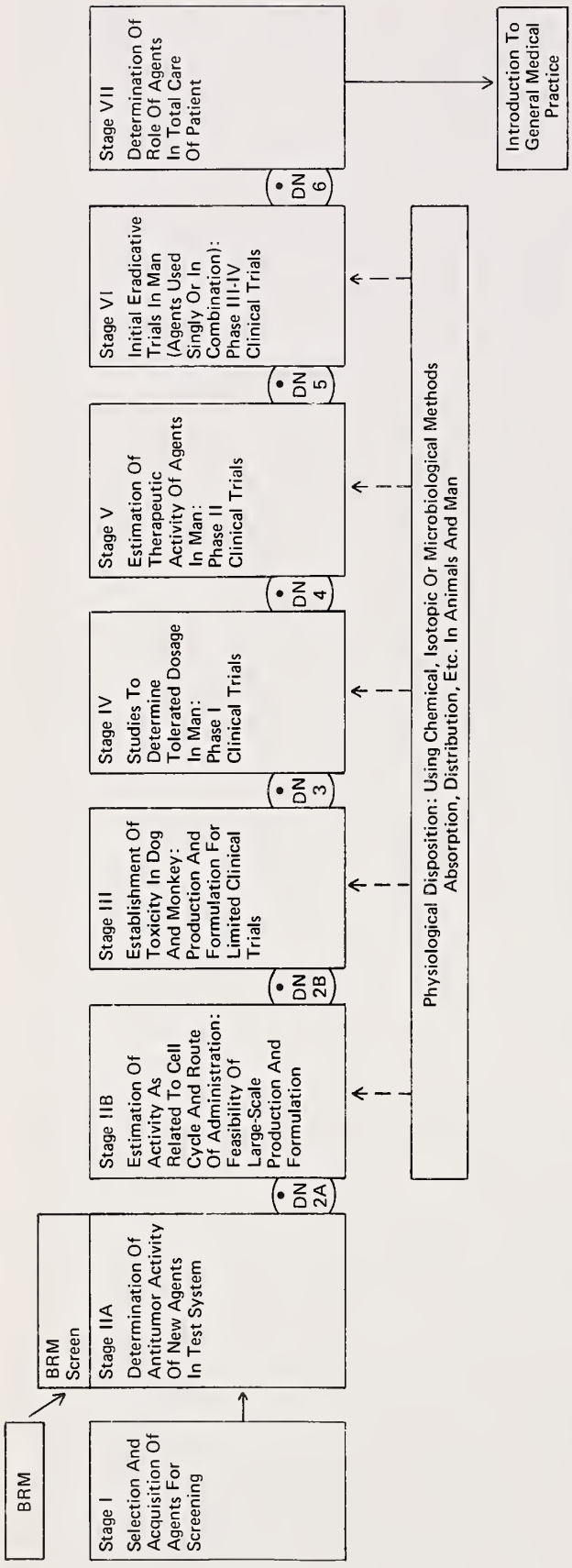

FIGURE A-7.—Linear array approach to clinical trials with BRM.

TABLE A-4.—*Division of Cancer Treatment BRM Program fiscal year 1980 funding (in thousands of dollars)*

Contracts	Fiscal year 1980, actual
HuLeIF	353
Antisera to immune IF	230
Immune IF standards	276
Standardized preparation of poly ICLC	286
Purchase of leukocyte IF	1,885
Purchase of fibroblast IF	2,071
Tumor-necrotizing factor	289
IF and lymphokine production at FCRF	414
Initiation of BRM Program Laboratory and clinical programs at FCRF	2,750
Development and production of clinical dosage	573
Characterization and analysis of proteinaceous materials	105
Phase I–II clinical evaluation of BRM	2,559
Chemoprevention of skin cancer	125
Planning and support services	80
Total contracts	11,996
Total new grants	(1,540)
Total BRM Program (including grants)	11,996 (13,536)

therapy. These initiatives were approved by the Board of Scientific Counselors for 1981; however, budgetary support for these projects had not been clearly identified. Program emphasis in the clinical area was on the IF, but increased support for monoclonal antibody and lymphokine trials is now anticipated. We expect a major effort in the use of monoclonal antibody as a carrier for toxic compounds in an attempt to increase the specificity of cancer therapy. The use of lymphokines for manipulation of the immune response and of cytokines (growth and maturation factors) for direct control of tumor growth and metastasis are expected to have major emphasis in the clinical program in the next few years.

PROGRAM PROJECTIONS

Extramural Program

The budget for fiscal year 1981 is shown in table A-7. The BRM Subcommittee developed a list with about 38 projects given higher priority and 5 a lower priority. The Program established an approach to most of these high priority areas either through programs funded in 1980 or through those that were initiated in 1981 and 1982. Our plan is to attempt to provide a stimulus for further investigation in as many of these high priority areas as possible. New contracts initiated in 1981–82 are shown in table A-7. The RFA by the BRM Program in 1982 are shown in table A-8. Program announcements for 1981–82 (table A-9) are continuing to prompt new grant applications in these high priority areas. With these initiations, many of the Subcommittee recommendations have been approached.

TABLE A-5.—*Clinical task orders for fiscal year 1980*

Institution	Agent	Approximate No. of patients		Amount of funding
		Phase I	Phase II	
Georgetown University Medical Center	Leukocyte IF	30	30	
Sidney Farber Cancer Institute	" "	"	"	
Northern California Cancer Program	" "	"		
Sloan-Kettering Cancer Institute	Fibroblast "	"	50	
University of Wisconsin	" "	"	30	
University of California at Los Angeles	Lymphoblast IF	"	"	
Duke University Medical Center	" "	"	60	
Subtotal				$1,396,519
University of California at San Diego	Thymosin	80		
The Fred Hutchinson Cancer Research Center	"	"		
George Washington University Medical Center	"	40		
Memorial Sloan-Kettering Cancer Institute	"	"		
Northern California Cancer Program	"	"		
Subtotal				969,466
Vanderbilt University	MVE-2	35		
Ohio State University	"	"		
Subtotal				192,673
Total		560	230	$2,558,658

Intramural Program

We used the fiscal year 1980 support to develop the BRM Program at the FCRF for renovations of interim laboratory space, purchase of major equipment, assistance in the renovation of the BRM Program building, purchase of supplies, and initial operating expenses. A building at the FCRF was renovated in 1981–82, and it now provides permanent space for the intramural laboratory program. Funds were also necessary for establishment of the clinical

TABLE A-6.—*Institutions with clinical master contracts with the BRM Program*

University of California at Los Angeles	University of Minnesota
University of California at San Diego	Northern California Cancer Program
University of Cincinnati	Ohio State University
Dartmouth College	Ontario Cancer Institute
Duke University	University of Pittsburgh
Fox Chase Cancer Center	Roswell Park Memorial Institute
The Fred Hutchinson Cancer Research Center	Sidney Farber Cancer Institute
Georgetown University Medical Center	Sloan-Kettering Institute
George Washington University Medical Center	University of Southern California
Hahnemann Medical School	Temple University (Southeastern Study Group)
Illinois Cancer Council	University of Texas (M. D. Anderson Hospital and Tumor Institute)
Institut de Cancerlogie et Immunogénétique, Villejuif, France	Vanderbilt University
Mayo Clinic	Wayne State University
	University of Wisconsin

TABLE A-7.—*Division of Cancer Treatment BRM Program, fiscal year 1981*

Contracts		Thousands of dollars
Purchase of:		
Lymphoblast IF		$ 2,112
Type II human IF		270
Leukocyte IF		93
Characterization and analysis of proteinaceous materials		105
Collection, storage, and quality assurance of BRM		285
FCRF Contract		1,914
Clinical support	$ 696	
Laboratory support	768	
BRM Program Screening Program	450	
Total contracts		4,779
Laboratory (intramural)		304
Total BRM Program		$ 5,083

TABLE A-8.—*RFP: 1981–82*

Proposal
Procurement of mouse IF
Chemical coupling of cytotoxic agents to tumor-reactive monoclonal antibody
Production of human lymphokines and cytokines
Studies on the immunogenicity of human cytokines in the mouse and the production of hybridomas secreting antibodies reactive specifically with the cytokine

TABLE A-9.—*BRM Program grant initiatives, 1981–82*

RFA, 1981–82
Monoclonal antibody in animal tumor models
Monoclonal antibody in cancer therapy
Animal tumor models for antipeptide growth factor and maturation factor therapy
Therapeutic use of lymphokines in cancer
Program announcements, 1981–82
Development of genetic engineering of BRM
Development of cell lines producing BRM
Effect of growth factors and antigrowth factors on cancer
Immunogenicity of purified TAA

unit at Frederick Memorial Hospital, for renovations of the outpatient unit, and the upgrading of the Radiology Department. Payment for assistance from the pathology and pharmacy services at the Hospital was also required.

Investigations into the production of monoclonal antibody, isolation and purification of TAA, production and purification of lymphokines and cytokines, and investigations into the basic mechanisms of actions of BRM are underway. The clinical unit at Frederick Memorial Hospital is now operational, and patients are being treated with genetically engineered IF and other BRM.

In late 1981, Dr. DeVita made a major decision to transfer the Laboratory of Immunodiagnosis, headed by Dr. Ronald Herberman, to the Division of Cancer Treatment. The subsequent decision to merge this Laboratory with the BRM Program was made by the Division. (The Laboratory of Immunodiagnosis had been a major laboratory in the Division of Cancer Biology and Diagnosis for many years.) The merger of this Laboratory with the intramural branch of the Program nearly doubled the size of the intramural research operations at the FCRF. The merger of the 2 groups was completed, and all of Dr. Herberman's group is colocated with the BRM Program at present. With this merger, Dr. Herberman became the Branch Chief in charge of intramural operations for the BRM Program. All the personnel from the Laboratory of Immunodiagnosis were incorporated into existing or newly created sections within the intramural branch. With this merger, the Biological Development Branch underwent a name change and is now known as the Biological Therapeutics Branch. The merger was completed by the Spring of 1982 with a fully integrated intramural research program moving into the newly renovated Building 567 and continuing some of its activities in current BRM Program space in Building 560 at the FCRF.

The Program was located in Frederick for various reasons: 1) The availability of space for the laboratories was a major consideration. 2) Clinical space at the Frederick Memorial Hospital was also an important factor. 3) A particular strength of the FCRF is that many of the investigators there are involved in ongoing research projects relevant to the BRM Program. Because of the existence of such ongoing research, decision makers considered it advantageous to establish collaborations with the existing groups at the FCRF rather than attempt to develop a broader and more expensive intramural program within the BRM Program. These interprogram collaborations will result in cost-effective research and may ultimately be a great benefit in making resources available through the Program.

FUTURE PERSPECTIVES

The BRM screening program is now operating at 75–100% capacity (*38*). An ongoing, functional program of evaluation and translation of potential BRM into clinical trials should be apparent in 1982. Given the required resources, the BRM Program should be able to accomplish on schedule most of the objectives set for it by the BRM Subcommittee.

The BRM extramural clinical trials are being conducted, and evaluated results should be available in 1982. A continued emphasis on extramural support of grants and contracts is a high priority of the Program. With the intramural laboratory and clinical program underway, by mid-1982 all aspects of the BRM Program should be functional to some extent.

That the rate of change in biology is rapidly accelerating is most apparent in the BRM area with respect to the development of new cancer therapies. New surgical approaches are being developed, and new forms of delivery for therapeutic radiation are available. Radiosensitizers are also being tested. Similarly, new chemotherapeutic agents and new methods of using existing agents are being developed. However, the use of BRM either alone or in combination with these other modalities is an area that is changing most rapidly. We can expect BRM to help in our achieving a greater understanding of cancer biology and a greater therapeutic specificity in the treatment of cancer patients in this decade (*42, 43*).

Given the new technology of the 1980's, this is an exciting time. We hope that sufficient resources will be available to support continuing efforts to develop biologicals into effective anticancer agents. As depicted by figure A-8, the rapidly advancing technology in computers, cell fusion, and genetic engineering all interrelate through the underpinnings of molecular biology to offer us an unparalleled opportunity in biological research that should translate into more effective treatments for cancer in this decade.

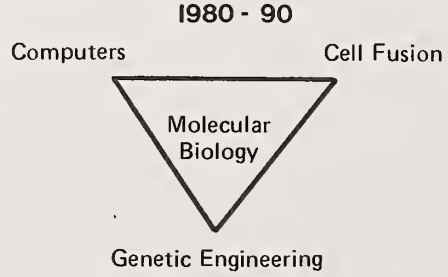

FIGURE A-8.—Technology transfer, 1980–90.

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ABBREVIATIONS

ADCC = antibody-dependent cellular toxicity
ALL = acute lymphocytic leukemia
AML = acute myelogenous leukemia
ara-C = cytosine arabinoside

BACON = bleomycin, adriamycin, 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea, oncovorin, and nitrogen mustard
BCNU = 1,3-bis(2-chloroethyl)-1-nitrosourea
BRM = biological response modifier(s)
BSA = bovine serum albumin

CAMF = Cytoxan, adriamycin, methotrexate, and 5-fluorouracil
CEA = carcinoembryonic antigen
CFU = colony-forming units
CHOP = cyclophosphamide, adriamycin, vincristine, and prednisone
CLL = chronic lymphocytic leukemia
CMC = carboxymethyl cellulose
CMI = cell-mediated immunity
CML = chronic myelocytic leukemia
CMV = cytomegalovirus
CNS = central nervous system
Con A = concanavalin A
COP = cyclophosphamide, vincristine, and prednisone
CPE = cytopathogenic effect
cpm = counts per minute
CPP = cyclophosphamide
CSA = colony-stimulating activity
CSF = colony-stimulating factor(s)
CTEP = Cancer Therapy Evaluation Program
CWS = cell wall skeleton(s)

DF = differentiation factor(s)
DMBA = 7,12-dimethylbenz[*a*]anthracene
DMH = dimethylhydrazine
DMSO = dimethyl sulfoxide
DNCB = dinitrochlorobenzene
DRB = 5,6-dichloro-1-β-D-ribofuranosyl-benzimidazole
DTC = diethyldithiocarbamide disodium

DTH = delayed type hypersensitivity
DTIC = dacarbazine; 5-(3,3-dimethyl-1-triazenyl)-1*H*-imidazole-4-carboxamide

EAC = erythrocyte-antibody-complement
EBV = Epstein-Barr virus
ED_{50} = mean effective dose
EGF = epidermal growth factor(s)
ELISA = enzyme-linked immunosorbent assay

FAC = 5-fluorouracil, adriamycin, and cyclophosphamide
FANFT = *N*-[4(5-nitro-2-furyl)-2-thiazolyl]formamide
FCRF = Frederick Cancer Research Facility
FTS = facteur thymique serique
5-FU = 5-fluorouracil

GM = granulocyte–macrophage (with CSF)
GVH = graft-versus-host

HBLA = human B-lymphocyte antigen
HCA = hydrocortisone acetate
HEPES = 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HGPRT = hypoxanthine-guanine phosphoribosyltransferase
HLC = human leukocyte culture(s)
HS = human serum
HTLA = human T-lymphocyte antigen
HuLeIF = human leukocyte interferon

Ia = immune response associated (IaDR)
IF = interferon(s)
γ-IF = immune interferon
IL-2 = interleukin-2
IND = investigational new drug
INH = isoniazid

LAI = leukocyte adherence inhibiton
LPS = lipopolysaccharide(s)

MAF = macrophage activating factor(s)
MCA = 3-methylcholanthrene
MDP = muramyl dipeptide(s)
2-ME = 2-mercaptoethanol
Me-CCNU = 1-(2-chloroethyl)-3-(4-methylcyclohexyl)-1-nitrosourea
MER = methanol extraction residue
MHC = major histocompatibility complex
MIA = migration inhibitory activity

MIF = macrophage migration inhibition factor(s)
MLC = mixed lymphocyte culture(s)
MLR = mixed leukocyte reaction(s)
MNU = *N*-methyl-*N*-nitrosourea
mol wt = molecular weight(s)
MTD = maximum tolerated dose(s)
MTX = methotrexate
MVE-2 = fraction of copolymer of maleic anhydride and divinyl ether (a low-molecular weight fraction of pyran)

NCI = National Cancer Institute
NGF = nerve growth factor(s)
NK = natural killer [cell(s)]

OH-BBN = *N*-butyl-*N*-4-hydroxybutyl nitrosamine

PAGE = polyacrylamide gel electrophoresis
PBL = peripheral blood lymphocyte(s)
PBS = phosphate-buffered saline
PDGF = platelet-derived growth factor(s)
PFC = plaque-forming cells
PGE = prostaglandin(s) of the E type
PHA = phytohemagglutinin
PMA = phorbol myristic acetate
PMN = polymorphonuclear
poly I:C = polyinosinic; polycytidylic (acids)
poly ICLC = poly I:C stabilized with poly-L-lysine
PPD = purified protein derivative
PWM = pokeweed mitogen

RES = reticuloendothelial system
RFA = Request for Application
RFP = Request for Proposal
RIA = radioimmunoassay(s)

SDS–PAGE = sodium dedecyl sulfate–polyacrylamide gel electrophoresis
SF = serum factor(s)
SGF = sarcoma growth factor(s)
SLE = systemic lupus erythematosus
SRBC = sheep red blood cells
SV40 = simian virus 40

TAA = tumor-associated antigen(s)
TCGF = T-cell growth factor(s)

TdT = terminal deoxynucleotidyl transferase

TF = transfer factor(s)

TFX = thymic factor X

TGF = transforming growth factor(s)

THF = thymic humoral factor(s)

TL = thymus leukemia

TNF = tumor necrosis factor(s)

TP I, II = thymopoietin I or II

TPA = 12-*O*-tetradecanoylphorbol-13-acetate

TRF = T-cell replacing factor(s)

TS = thymostimulin

TSA = tumor-specific antigen(s)

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